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ENCAPSULATION OF ROSMARINIC ACID INTO BIOPOLYMER-BASED MICROPARTICLES FOR TOPICAL DELIVERY

DISSERTATION FOR MASTER DEGREE IN BIOENGENEERING
SPECIALIZATION IN BIOLOGICAL ENGINEERING

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"Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar.
Al andar se hace el camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.
Caminante no hay camino
sino estelas en la mar."

Antonio Machado

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"I can no other answer make but thanks, and thanks, and ever thanks."

William Shakespeare

ABSTRACT

Antioxidants constitute important cosmetic active ingredients capable of both protecting skin cells against the damaging effects of reactive species, as well as protecting cosmetic formulations against oxidative degradation. Recently interest has increased in finding natural antioxidants for cosmetic applications due to their strong activities and non-toxicity. Rosmarinic acid (RA) is a naturally occurring phenolic compound with a number of interesting biological activities, including antioxidant, anti-carcinogenic, anti-inflammatory, amongst others, and is therefore a compound of interest for cosmetic applications. However transdermal delivery of RA through cosmetic formulations is a challenge due to various reasons, including instability, poor solubility in water and low partition coefficient, constraining the transport across biological barriers, the inclusion in a cosmetic formulation and the efficacy of the antioxidant. To circumvent these drawbacks microencapsulation technology has been proposed as a delivery system to increase stability, protect against degradation and to direct and control the release of active ingredients. Many encapsulation methods are described in the literature, but one of the most used in industrial applications is the spray-drying process, due to its low cost, availability of equipment and efficiency. Biopolymers and biodegradable polymers, such as chitosan, are the encapsulating materials with greater interest for cosmetic applications.

The purpose of this study was to prepare and characterize chitosan and modified chitosan microparticles encapsulating RA by a spray drying technique, as means to overcome its limitation in cosmetic formulations, as well as to study the controlled release of RA from the particles under cosmetic formulation conditions (water and oil) and evaluate the antioxidant capacity. High performance liquid chromatography (HPLC) and UV-Vis spectrometry methods for RA determination were developed and validated. The methods presented good linearity with correlation coefficients greater than 0.999, and good precision results with coefficients of variation values lower than 5%. The limits of detection were 0.02 $mg.L^{-1}$ for HPLC and 0.38 $mg.L^{-1}$ and 0.25 $mg.L^{-1}$ for UV-Vis spectrometry in water and oil, while the limits of quantification were 0.08 $mg.L^{-1}$, 1.27 $mg.L^{-1}$ and 0.83 $mg.L^{-1}$, respectively. We concluded that is possible to encapsulate RA using different types of chitosan, through a spray-drying process. Satisfactory product yields of 42.6% and 39.8%, and association efficiencies of 92.6% and 59.6% were obtained for chitosan and modified chitosan particles. Spherical particles with mean diameters of 4.2 and 7.7 µm were obtained for chitosan and modified chitosan. Controlled release studies of RA showed a fast release (100% at 30 min) for modified chitosan particles in water, and a slower release (75% at 2 h) in oil. Chitosan particles showed a fast release (90% at 45 min) in both mediums. Antioxidant assessment showed that chitosan-based microparticles did not compromise RA good antioxidant activity performance. As preliminary tests, the results in this study are significant and prove the success of RA microencapsulation for a topical delivery, but further studies need to be performed to obtain a more controlled and sustained release system.

Keywords: Microencapsulation, rosmarinic acid, chitosan, controlled release, topical delivery

RESUMO

Antioxidantes constituem importantes ingredientes ativos cosméticos capazes de proteger as células da pele contra os efeitos nocivos de espécies reativas, e de proteger as formulações cosméticas da degradação oxidativa. Atualmente o interesse em encontrar antioxidantes naturais para aplicações cosméticas tem aumentado, devido à sua forte atividade e não-toxicidade. O ácido rosmarínico (RA) é um composto fenólico natural com diversas propriedades de interesse: antioxidante, anti-cancerígena, anti-inflamatória, etc., sendo um composto de interesse para aplicações cosméticas. No entanto, o transporte transdérmico do RA através de formulações cosméticas é um desafio, devido à instabilidade, baixa solubilidade em água e baixo coeficiente de partição, restringindo o seu transporte através de barreiras biológicas, a inclusão em formulações cosméticas e a eficácia do antioxidante. Para contornar estas questões têm sido propostas tecnologias de microencapsulação, que permitem melhorar a estabilidade, proteger contra a degradação e controlar e dirigir a libertação de compostos bioativos. Têm sido descritos diversos métodos de encapsulação, mas um dos mais usados em aplicações industriais é o processo de secagem por atomização, devido ao baixo custo, disponibilidade de equipamento e eficiência. Biopolímeros e polímeros biodegradáveis, como o quitosano, são os materiais encapsulantes de maior interesse para aplicações cosméticas.

O objetivo deste estudo foi preparar e caracterizar micropartículas de RA encapsulado por quitosano e quitosano modificado pela técnica de secagem por atomização, como forma de transpor as suas limitações em aplicações cosméticas, assim como estudar a libertação controlada do RA sob condições de formulações cosméticas (água e óleo) e avaliar a capacidade antioxidante. Métodos de HPLC e espectroscopia UV-Vis para determinação do RA foram desenvolvidos e validados. Estes métodos apresentaram boa linearidade, com coeficientes de correlação superiores a 0.999, e boa precisão, com coeficientes de variação inferiores a 5%. Os limites de deteção foram 0.02 $mg.L^{-1}$ para HPLC e 0.38 $mg.L^{-1}$ e 0.25 $mg.L^{-1}$ para espectroscopia, em água e óleo, enquanto os limites de quantificação foram 0.08 $mg.L^{-1}$, 1.27 $mg.L^{-1}$ e 0.83 $mg.L^{-1}$, respetivamente. Concluiu-se que é possível encapsular RA usando diferentes tipos de quitosano por secagem por atomização. Foram obtidos rendimentos de 42.6% e 39.8%, e eficiências de associação de 92.6% e 59.6%, para as partículas de quitosano e quitosano modificado. Produziram-se partículas esféricas com diâmetros médios de 4.2 e 7.7 µm. Os estudos de libertação controlada mostram uma rápida libertação (100% após 30 min) para as partículas de quitosano modificado em água, e uma libertação mais lenta (75% após 2 h) em óleo. As partículas de quitosano mostraram uma rápida libertação (90% aos 45 min) nos dois meios. A avaliação da atividade antioxidante mostrou que a encapsulação em partículas de quitosano não comprometeu a boa capacidade antioxidante do RA. Como testes preliminares os resultados obtidos são significativos e provam o sucesso da encapsulação de RA para aplicação tópica, mas mais estudos devem ser realizados para se obter um sistema de libertação mais controlado.

Palavras-chave: Encapsulação, ácido rosmarínico, quitosano, libertação controlada, aplicação tópica

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GLOSSARY

A	Peak area
Abs	Absorbance
ABTS	2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)
AE	Association efficiency
AHA	Alpha hydroxy acid
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
C	Concentration
CA	Caffeic acid
CAS	Chemical Abstracts Service
CD	Cyclodextrin
CoA	Coenzyme A
CO_2	Carbon dioxide
CV	Coefficient of variation
DAD	Diode-array detection
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
GSH	Glutathione
HIV-1	Human immunodeficiency virus type one
HPLC	High-Performance Liquid Chromatography
IUPAC	International Union of Pure and Applied Chemistry
LM	Light microscope
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass Spectrometry
NLC	Nanostructured lipid carriers
NP	Nanoparticles
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCL	Polycaprolactone
PEG	Polyethylene glycol
PLA	Poly(lactic acid)
PLGA	Poly(lactic co-glycolic acid)
QSPR	Quantitative structure-permeability relationships
RA	Rosmarinic acid
ROS	Reactive oxygen species
SEM	Scanning electron microscope
SLN	Solid lipid nanoparticles
TE	Trolox equivalents
UV	Ultraviolet radiation
UV (HPLC-UV)	UV detection

CHAPTER 1: INTRODUCTION

1.1. Background

Microencapsulation has been widely explored by the pharmaceutical, food, cosmetic, textile, personal care, chemical, biotechnology and biomedical industries. There are numerous possibilities to use microencapsulation as a technique to obtain products with high added value and therefore widespread interest has developed in microencapsulation technology (Hammad et al*.,* 2011; Dubey et al., 2009; Wesselingh et al*.*, 2007; Ghosh, 2006; Benita, 2005).

The sector of cosmetics and personal care products has been evaluated in multi-billion dollars internationally and has shown great expansion in the global market. To have success in such a competitive and demanding sector, the products must differentiate (Euromonitor, 2011; Michael, 2009). The skin-care and cosmetic formulators are being challenged to develop efficacious and clearly distinctive topical formulations and also include new bioactive ingredients, which can be achieved by means of using emergent technologies, such as microencapsulation. Numbers of materials as cosmetic active ingredients are being investigated such as antioxidants, vitamins, sun filters and natural plant extracts, namely essential oils and natural compounds (Kim et al., 2010). Meanwhile many of these cosmetic and personal care active ingredients are unstable and sensitive to temperature, pH, light and oxidation, and may undergo reactions that lead to the reduction or loss of its effectiveness or even to the degradation of the product. Thus, microencapsulation technologies have been proposed to increase stability, to protect against degradation, to direct and control the release of active ingredients used in cosmetic products, or even to mask undesired properties of the active components, such as their odour. In addition, the topical and transdermal delivery of cosmetic active ingredients requires safe, non-toxic and effective means to reach the target destination sites in the body. Therefore microencapsulation has been used in the development of cosmetic formulations that are more stable, more effective and with improved sensory properties, having found an increasing number of applications in this market (Pardeike et al., 2009; Soest, 2007; Sinko, 2006; Lumsdon et al., 2005; Rosen, 2005; Gallarate et al., 1999). Published patents in the area of microencapsulation suggest that both industrial and academic sectors are urging to explore this area, namely in the fields of cosmetics and personal care products for topical application (Conopco Inc. and D/B/A Unilever, 2010; Capsutech Ltd., 2009; Coreana Cosmetics Co. Ltd, 2001; Shaklee Corporation, 2001; Maybelline Intermediate Company, 1999; L'Oreal, 1998; Sunsmart, Inc. and Sibmicro Encapsulation Technologies, Inc., 1998; R.P. Scherer Corporation, 1996; Durand, 1995).

1.2. Cosmetic Ingredients

1.2.1. Antioxidants in Cosmetics

Antioxidants are molecules capable of inhibiting the oxidation of other molecules (Zheng et al., 2001). Although oxidation reactions are essential for life, they can also be damaging, leading to cell injury and death. Topically applied antioxidants constitute an important group of cosmetic active ingredients capable of preventing the occurrence and reducing the severity of skin damage and aging (Oresajo et al., 2012; Wang et al., 2008).

Skin, being the outermost barrier of the body, is frequently exposed to oxidative stress from exogenous sources (ultraviolet (UV) radiation, air pollutants, toxins) leading to the generation of reactive oxygen species (ROS) and free radicals. Owing to the presence of one unpaired electron in the outermost shell of the atomic nucleus, ROS and free radicals are highly reactive and have the affinity to either donate or obtain electrons from another species to achieve stability. This may lead to the oxidation and damage of biomolecules, including lipids, proteins and deoxyribonucleic acid (DNA). To counteract the harmful effects of free radicals, the skin possesses an antioxidant network responsible for maintaining the balance between these reactive species and antioxidants. The antioxidant network of the skin contains antioxidant species such as vitamin E, ubiquinones, carotenoids, vitamin C, uric acid and glutathione (Abla et al., 2013; Psotova et al., 2006). Intrinsic and extrinsic free radical formation cause oxidative stress, the major and most important cause of skin damage. Intrinsic free radical formation occurs when the ability of human skin cells to repair DNA damage steadily reduces with years and the antioxidative defence becomes less effective (Poljsak et al., 2013). On the other hand, antioxidants present in the skin are susceptible to UV exposure and a single suberythemal dose[1] can deplete their concentration in half, resulting in extrinsic free radical formation (Abla et al., 2013). Ionizing radiations, including UV radiation, are major environmental factors that dramatically alter skin homeostasis, causing a massive generation of cytotoxic reactive oxygen species and inducing DNA damage. Their cytotoxicity increases by interaction with membrane phospholipids, inducing peroxidative processes and the generation of lipoperoxy radicals (Sánchez-Campillo et al., 2009). Free radical formation and oxidative stress may lead to skin damage, premature skin aging, inflammatory reactions and malignant skin lesions (skin cancer) (Abla et al., 2013; Oresajo et al., 2012). Under these oxidative conditions the endogenous antioxidant system may be insufficient and exogenous agents with a strong antioxidant capacity could be beneficial (Sánchez-Campillo et al., 2009).

[1] Threshold *dose* of radiation which, applied to the skin, may produce an erythema (sunburn).

Antioxidants are compounds that can delay or inhibit the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Zheng et al., 2001). Antioxidants act either by an indirect mechanism where they form chelates or by a direct mechanism where they donate an electron to the reactive oxygen species (Abla et al., 2013). Inhibition of propagating radicals during the oxidative chain reaction is believed to be the dominant mechanism by which phenolic antioxidants operate. A mechanism proposed to account for this behaviour is given in Figure 1 (Servili et al., 2014; Zheng et al., 2001; Basaga et al., 1997).

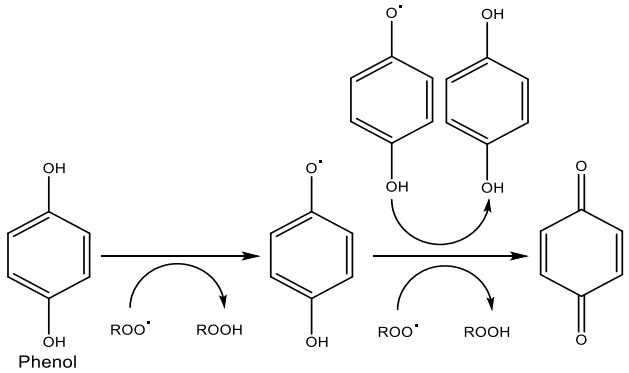


Figure 1. Example of the mechanism of the antioxidant activity of phenols.

Antioxidants protect skin cells against the damaging effects of reactive species, such as singlet oxygen, superoxide, peroxyl and hydroxyl radicals, thus preventing oxidative stress and skin aging and damage. Several studies concerning topically applied antioxidants have recently confirmed that antioxidant compounds can scavenge oxidative radicals, decrease lipid peroxidation and thus provide protection against oxidative stress. Therefore supplementing skin with antioxidants may strengthen the antioxidant capacity and reduce ROS induced skin damage (Abla et al., 2013).

Moreover cosmetic products, which commonly comprise fats, oils and lipid containing matrices, are often prone to oxidation reactions, which generate compounds that cause rancidity, off-aromas and activity losses (Carrillo et al., 2006; Basaga et al., 1997). Oxidative degradation of fats, oils and lipids is a major factor responsible for the deterioration of cosmetics and thereby for limiting the shelf life of these products. The use of antioxidants during the manufacturing process can provide protection against oxidative degradation of cosmetic products caused by free radicals (Wang et al., 2008; Aruoma et al., 1996).

A variety of antioxidants can be used in topical cosmetic products. These include vitamin C (ascorbic acid and its derivatives), vitamin E (tocopherol and its derivatives), ubiquinones and its derivatives, and glutathione (GSH) and its precursors. In addition, activators of several enzyme systems that regenerate these antioxidants, such as GSH peroxidases and GSH reductase, and enzymes that neutralize ROS, such as superoxide dismutases, catalase, and quinone reductase,

are also used topically to potentiate antioxidant systems within the skin. Plant-derived compounds such as carotenoids and phenols are an important group of compounds showing antioxidant properties. Dozens of polyphenols have been shown to have antioxidant and anti-inflammatory activities on the skin, and are currently used in skin care products (Oresajo et al., 2012). In general there are three basic categories of antioxidants: natural, synthetic and semi-synthetic. Recently, interest has increased considerably in finding naturally occurring antioxidants for cosmetic applications to replace synthetic ones, which are being restricted due to their carcinogenic effects (Zheng et al., 2001).

Nevertheless, the efficacy and benefit of an antioxidant is very much dependent on the delivery of the antioxidant to the organism. When formulating with antioxidants, compatibility is a major concern. Care must be taken to protect the antioxidant from neutralizing other cosmetic active ingredients or from being neutralized under the conditions of the formulation. Product stabilization is also crucial. Since antioxidants can be very unstable, they may become oxidized and inactive before reaching the target body site. Antioxidants must also be absorbed into the skin, reach their target tissue in the active form, and remain there long enough to exert the desired effects (Oresajo et al., 2012).

These issues can be solved by applying the microencapsulation technology, which provides the required technique for conversion of the antioxidant to an effective functional ingredient. The application of microencapsulated bioactive compounds as functional ingredients in cosmetic applications exhibits a significant potential, since it could enable the enrichment of various cosmetic products with natural antioxidants and its effective permeation through the skin. Owing to microencapsulated ingredients, the production of many products that were considered technically unfeasible has been enabled (Belščak-Cvitanović et al., 2011).

1.2.2. Natural sources of compounds of cosmetic interest

Herbs have been used for a large range of purposes including medicine, pharmaceuticals, nutrition, food preservation, flavourings, beverages, repellents, fragrances and cosmetics. Since prehistoric times, herbs were the basis for medicinal therapy until synthetic drugs were developed in the nineteenth century (Okoh et al., 2010; Zheng et al., 2001). In recent decades, the use of herbs and plants has been of great interest, as they have been the sources of natural products, commonly named as bioactive compounds (Zibetti et al., 2013; Wang et al., 2008). Bioactive compounds are natural active ingredients produced in plants as secondary metabolites that have an effect on a living organism, tissue or cell (Zibetti et al., 2013). The use of bioactive compounds from various herbs as functional ingredients in food, beverage and cosmetic

applications is gaining growing interest in the last few years (Belščak-Cvitanović et al., 2011). Natural matrices represent a rich source of biologically active compounds and are an example of molecular diversity, with recognized potential for the development of cosmetics or cosmeceuticals (Barroso et al., 2014).

Bioactive compounds of plant origin have been shown to have several beneficial properties. Nowadays the use of natural compounds is also increasing around the world due to their mild features and low side effects (Belščak-Cvitanović et al., 2011; Sánchez-Campillo et al., 2009; Erkan et al., 2008). Cosmetic preparations from herbal origin are popular among consumers, as these agents are typically non-toxic and possess strong activities (Barroso et al., 2014).

Herbs provide a wide spectrum of bioactive compounds with beneficial activities, namely polyphenols, vitamins, polysaccharides and minerals (Belščak-Cvitanović et al., 2011). Bioactive properties of various plants are connected with the presence of phenolic compounds, especially flavonoids. The biological, pharmacological and medicinal properties of this group of compounds have been extensively reviewed and related to their antioxidant properties by preventing UV induced oxygen free radical generation and lipid peroxidation. Many of these phytochemicals possess significant antioxidant capacities that have been associated with lower incidence and lower mortality rates of cancer (Zheng et al., 2001). Since oxidative stress is one of the major mechanisms for skin aging and damage, phytochemicals such as phenolic compounds could be useful for treating or preventing those conditions (Barroso et al., 2014; Luis et al., 2007). In order to prolong the storage stability of cosmetics and foods, synthetic antioxidants are used for industrial processing. But the toxicity effects of some synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have already been documented, and they include carcinogenic effects in living organisms (Türkoğlu et al., 2007). For this reason, governmental authorities and consumers are concerned about the safety and the potential effects of synthetic additives on health, and are demanding for natural ingredients. Preliminary studies demonstrated that some herbs extracts are as efficient as the synthetic antioxidants (Hernández-Hernández et al., 2009; Erkan et al., 2008; Wang et al., 2008). Phenolic compounds are also known to possess antimicrobial activity and are generally recognized as safe substances, therefore they are used to prevent post-harvest growth of native and contaminant bacteria (Okoh et al., 2010). For all the described reasons in recent years a lot of interest has been devoted to exploring antioxidants from natural sources (Psotova et al., 2006; Basaga et al., 1997).

Recently numerous reports have described antioxidants and compounds with beneficial activities present in fruits, vegetables, herbs and cereals extracts (Wang et al., 2008). Rosemary

(*Rosmarinus officinalis*), sage (*Salvia officinalis*), thyme (*Thymus vulgaris*) and lavender (*Lavendula angustifolia*) are native to the Mediterranean region and cultivated world-wide, and balm (*Melissa officinalis*) and spearmint (*Mentha spicata*) are common plants in Britain and other european countries. Researchers have found that these plants are a source of compounds possessing high antioxidant, anti-inflammatory, antimicrobial and anti-carcinogenic activities. In particular rosemary extracts possess very useful antioxidant properties, which appear to be related to their content of phenolic compounds, amongst which rosmarinic acid was found to be one of the most important (Hernández-Hernández et al., 2009; Erkan et al., 2008; Wang et al., 2004; Aruoma et al., 1996). Gachkar et al. (2007) reported the chemical composition, antibacterial, antioxidative and radical-scavenging properties of the essential oils of *Rosmarinus officinalis* and *Cuminum cyminum* obtained by steam distillation. The antimicrobial activity of essential oils obtained from oregano, thyme, sage, rosemary, clove, coriander, garlic and onion against both bacteria and fungi has also been reported (Okoh et al., 2010).

The inclusion of antioxidants, or rich sources thereof (including herb extracts), in cosmetics is becoming a common procedure of this industry, namely in the production of various cologne waters, body lotions and creams, sunscreens, bathing lotions, hair lotions, shampoos and as components of disinfectants and insecticides (Campos et al., 2012; Okoh et al., 2010; Sánchez-Campillo et al., 2009; Wang et al., 2004).

Rosemary - *Rosmarinus officinalis*

Rosmarinus officinalis (Figure 2), commonly known as rosemary, is a perennial herb that belongs to the Lamiaceae family (Sui et al., 2012; Okoh et al., 2010; Kim et al., 2001). It is native to the Mediterranean region in countries like Portugal, Spain, Morocco, Tunisia and Italy, but is now widely distributed and has been cultivated in many regions (Fernandes et al., 2013; Liu et al., 2011). Rosemary extracts have been used for thousands of years as flavourings, pharmaceuticals, alternative medicine and natural therapies (Couto et al., 2012; Zu et al., 2012; Fadel et al., 2011).

Figure 2. Rosemary (*Rosmarinus officinalis*) (The Herb Society of America, 2009).

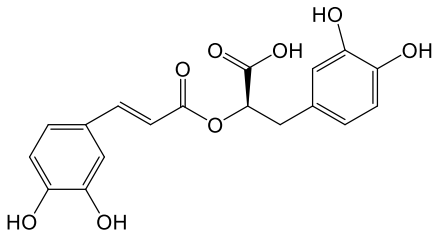
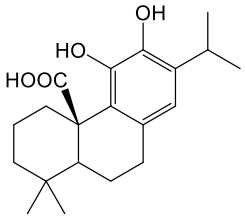
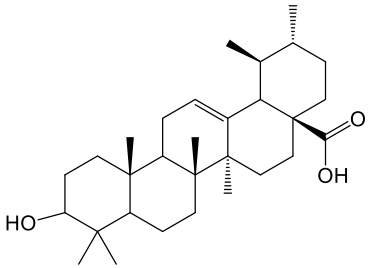
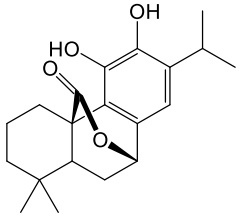
Recently rosemary has been largely studied as a source of natural products with diverse biological activities (Visentin et al., 2012). In addition to being used as a food flavouring, several extracts, essential oils and chemical constituents isolated from *Rosmarinus officinalis* demonstrated a number of interesting biological activities, including antioxidant, antimicrobial, anti-inflammatory, anti-tumorigenic, anti-allergic, metal chelating, chemo-preventive and cyto-protective activities (Couto et al., 2012; Sui et al., 2012; Liu et al., 2011; Wang et al., 2004), which makes them suitable candidates as bioactive ingredients to design functional cosmetic, pharmaceutical and food products (Visentin et al., 2012; Zu et al., 2012; Psotova et al., 2006).

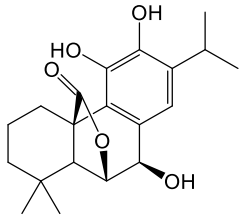
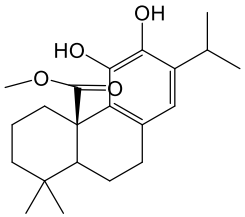
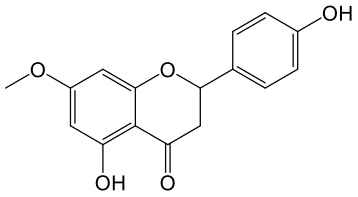
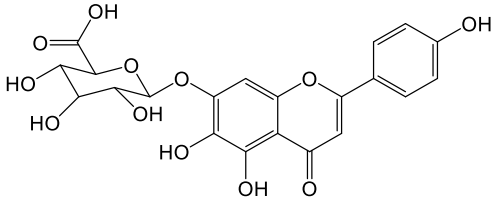
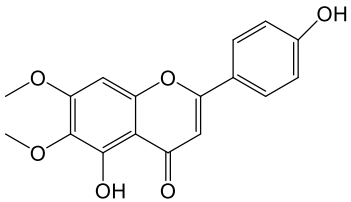
R. officinalis has been widely accepted as one of the spices with highest antioxidant activities of all the herbs and spices that have been investigated (Wang et al., 2008). The antioxidant activity of rosemary is related to rosemary's content of polyphenolic compounds (Couto et al., 2012; Del Bano et al., 2003). The compounds associated with the antioxidant activity of the herb are the phenolic acids such as rosmarinic acid, ursolic acid and caffeic acid, and the phenolic diterpenes such as carnosoic acid, carnosol, rosmanol, epirosmanol and methyl carnosate (Table 1) (Zibetti et al., 2013; Carvalho et al., 2005¸ Del Bano et al., 2003). In addition, several flavonoids, such as genkwanin, scutellarein, cirsimaritin and luteolin have been identified (Table 1) (Del Bano et al., 2003). Several studies have indicated that the most active antioxidant compounds in rosemary are the diterpene, carnosic acid, and the phenolic acid, rosmarinic acid, which are the most abundant constituents and also well known for their various biological properties (Sui et al., 2012; Liu et al., 2011; Luis et al. 2007). Rosmarinic acid is the major and most important phenolic component of the plant and is therefore considered a chemical marker of this species. It exhibits a wide spectrum of biological activities, mainly antioxidant and anti-carcinogenic (Couto et al., 2012; Psotova et al., 2006; Wang et al., 2004).

Rosmarinic acid

Rosmarinic acid (RA) is a naturally occurring phenolic compound commonly found in plants belonging to the Boraginaceae family and the subfamily Nepetoideae of the Lamiaceae family (Campos et al., 2014; Sánchez-Campillo et al., 2009). RA was originally isolated in 1958 from the rosemary plant (*Rosmarinus officinalis*). Some of the species from which this compound has been reported include rosemary (*Rosmarinus officinalis)*, sage (*Salvia* officinalis), peppermint (*Mentha piperita*), thyme (*Thymus vulgaris*), marjoram (*Origanum vulgare)*, basil (*Ocimum basilicum*) and Prunella (*Prunella vulgaris)* (Hossan et al., 2014; Kim et al., 2010).

Table 1. Properties and biological activities of rosemary (*Rosmarinus officinalis*) antioxidants.

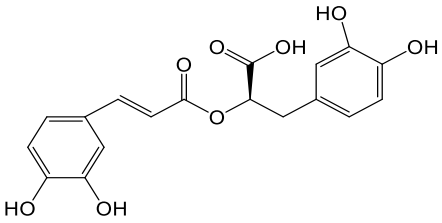
Compound IUPAC Name CAS Number Molecular Formula	Chemical Structure	Natural Occurrences	Solubility ($mg.mL^{-1}$)	Biological activities
Rosmarinic acid (2R)-3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxypropanoic acid 20283-92-5 $C_{18}H_{16}O_8$		*Rosmarinus officinalis* *Salvia officinalis* *Mentha piperita* *Thymus vulgaris* *Origanum majorana* *Ocimum basilicum* *Prunella vulgaris*	Ethanol: 25 DMSO: 25 DMF: 25 PBS: 15 Water: 1.0	Antioxidant Anti-inflammatory Anti-bacterial Anti-viral Anti-carcinogenic Anti-angiogenic Anxiolytic Neuro-protective
Carnosic acid (4aR,10aS)-5,6-Dihydroxy-1,1-dimethyl-7-propan-2-yl-2,3,4,9,10,10a-hexahydrophenanthrene-4a-carboxylic acid 3650-09-7 $C_{20}H_{28}O_4$		*Rosmarinus officinalis* *Salvia officinalis*	Ethanol: 30 DMSO: 30 DMF: 30 Water: 1.0	Antioxidant Anti-microbial Anti-angiogenic Anti-carcinogenic
Ursolic acid (1S,2R,4aS,6aR,6aS,6bR,8aR,10S,12aR,14bS)-10-hydroxy-1,2,6a,6b,9,9,12a-heptamethyl-2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid 77-52-1 $C_{30}H_{48}O_3$		*Rosmarinus officinalis* *Mirabilis jalapa* *Lavandula spica* *Mentha piperita* *Ocimum basilicum*	Ethanol: 5.0 DMSO: 25 DMF: 25	Antioxidant Anti-carcinogenic Anti-angiogenic Cardio-protective
Carnosol (7β)-11,12-Dihydroxy-7,20-epoxyabieta-8,11,13-trien-20-one 5957-80-2 $C_{20}H_{26}O_4$		*Rosmarinus officinalis* *Salvia pachyphylla*	Ethanol: 8.0 DMSO: 25 DMF: 35 PBS: 0.030 Water: 0.0014	Antioxidant Anti-inflamatory Anti-angiogenic Anti-carcinogenic

Rosmanol (6β,7β)-7,11,12-Trihydroxy-6,20-epoxyabieta-8(14),9(11),12-trien-20-one 80225-53-2 $C_{20}H_{26}O_5$		*Rosmarinus officinalis*	n.d.	Antioxidant Anti-inflamatory Anti-carcinogenic
Methyl carnosate methyl (4aR,10aS)-5,6-dihydroxy-1,1-dimethyl-7-propan-2-yl-2,3,4,9,10,10a-hexahydrophenanthrene-4a-carboxylate 82684-06-8 $C_{21}H_{30}O_4$		*Rosmarinus officinalis* *Salvia officinalis*	n.d.	Antioxidant Anti-bacterial
Genkwanin 5-hydroxy-2-(4-hydroxyphenyl)-7-methoxychromen-4-one 437-64-9 $C_{16}H_{12}O_5$		*Rosmarinus officinalis* *Alnus glutinosa* *Notholaena bryopoda* *Asplenium normale* *Teucrium ramosissimum*	Soluble in: DMSO, DMF	Antioxidant Anti-carcinogenic
Scutellarin 5,6,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one 27740-01-8 $C_{21}H_{18}O_{12}$		*Rosmarinus officinalis* *Scutellaria barbata* *Scutellaria lateriflora*	Soluble in: alkali hydroxides, glacial acetic acid DMSO: 1.0	Antioxidant Anti-carcinogenic
Cirsimaritin 5-hydroxy-2-(4-hydroxyphenyl)-6,7-dimethoxychromen-4-one 6601-62-3 $C_{17}H_{14}O_6$		*Rosmarinus officinalis* *Teucrium ramosissimum* *Lemon verbena* *Cirsium vulgare*	n.d.	Antioxidant Anti-carcinogenic

n.d.: not described; IUPAC: International Union of Pure and Applied Chemistry; CAS: Chemical Abstracts Service; DMSO: Dimethyl sulfoxide; DMF: Dimethyl formamide; PBS: Phosphate buffered saline

Rosmarinic acid (Table 2) is formally known as (2R)-3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4 dihydroxyphenyl)prop-2-enoyl]oxypropanoic acid (Hossan et al., 2014). It is a white-yellow powder slightly soluble in water, but well soluble is most organic solvents (Kim et al., 2010).

Table 2. Chemical and physical properties of rosmarinic acid.

Compound (Acronym)	Rosmarinic acid (RA)
IUPAC Name	(2R)-3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxypropanoic acid
CAS Number	20283-92-5
Molecular Formula	$C_{18}H_{16}O_8$
Chemical Structure	
Molar mass ($g.mol^{-1}$)	360.31
Melting point (°C)	173
Density	1.55
Vapor pressure (mm Hg)	1.10×10^{-13}
log Kow	1.82
pKa	3.57
UV absorption max (nm)	221, 291, 330
Solubility ($mg.mL^{-1}$)	Ethanol: 25 DMSO: 25 DMF: 25 PBS: 15 Water: 1.0

Chemically, RA is an ester of caffeic acid (CA) and 3,4-dihydroxyphenyllactic acid, being a CA dimer formed by esterification (ester condensation) of CA and its hydrated derivative 3,4-dihydroxyphenyllacetic acid. Biologically, the biosynthesis of RA starts with precursor molecules L-phenylalanine and L-tyrosine and uses 4-coumaroyl-CoA (coenzyme A) from the general phenylpropanoid pathway as hydroxycinnamoyl donor (Appendix A: Biosynthetic pathway of rosmarinic acid) (Petersen et al., 2013, 2009, 1993; Sundaram et al., 2010). In plants, RA acts as a preformed constitutively accumulated defence compound (Sánchez-Campillo et al., 2009).

Rosmarinic acid was reported to have a number of interesting biological activities (Campos et al., 2014). RA has anti-inflammatory and anti-allergic properties, inhibiting several inflammatory processes by direct effects on T cells (Stansbury et al., 2012; Sánchez-Campillo et al., 2009). This compound possesses antiviral, including against herpes simplex virus and human immune-deficiency virus type one (HIV-1), and antibacterial activities (Gudzenko, 2013; Fecka et al., 2007).

Rosmarinic acid was reported to possess notable activity against *Bacillus subtilis* and *Escherichia coli*. It has also been reported to possess activity against *Pseudomonas aeruginosa, Shigella sp, Staphylococcus aereus, Enterobacter, Candida albicans* and *Aspergillus niger*. In vivo studies have shown that RA also exhibits anti-angiogenic, anxiolytic, anti-atherosclerotic, anti-fibrotic, chemo-protective and neuro-protective activities, as well as reduction of atopic dermatitis and prevention of Alzheimer's disease (Bhatt et al., 2014; Silva et al., 2014b; Gudzenko, 2013; Kelsey et al., 2010; Swarup et al., 2007; Sanbongi et al., 2004, Hooker et al., 2001). However the most important activities of RA are the antioxidant and anti-carcinogenic effects (Campos et al., 2014). RA has a potent antioxidant activity, stronger than that of vitamin E, by scavenging oxygen free radicals, delaying vitamin E depletion, preventing the oxidation of low density lipoproteins and providing photo-protection of keratinocytes (Kim et al. 2010; Sánchez-Campillo et al., 2009). RA helps to prevent cell damage caused by free radicals, thereby reducing the risk of cancer (Kim et al., 2010). RA has been found to be effective against a number of cancer cell lines, including skin cancer and melanoma, colon cancer, leukaemia and breast cancer (Osakabe et al., 2004). The effects of skin cancer have been reversed by topical application of RA, which acted against skin tumours by exerting an anti-inflammatory and antioxidant effect. RA was found to have dual anti-carcinogenic capability, namely that of protecting normal cells against radiation but also of sensitizing melanomas to radiation (Alcaraz et al., 2014; Hossan et al., 2014).

Rosmarinic acid is therefore a compound of interest for cosmetic applications. However, transdermal delivery of this antioxidant through cosmetic formulations, such as emulsions and creams, is a challenge due to instability, discoloration, poor solubility in water and low partition coefficient, constraining the transport across biological barriers. The technological handling of such type of compounds is sometimes limitative due to their reactivity, so their incorporation in a cosmetic formulation becomes difficult if they are not duly protected from the interactions to which they are exposed when incorporated in cosmetic matrices. The solubility characteristics of the antioxidant in relation to the site of action must also be considered (Campos et al., 2014, 2012; Silva et al., 2014b; Visentin et al., 2012; Kim et al., 2010). In view of the above mentioned drawbacks, encapsulation with an appropriate carrier material for RA delivery, protection and release is necessary to obtain an effective product (Silva et al., 2014b). Encapsulation will protect the antioxidant during manufacturing processes, improve its long-term stability, its permeability characteristics through the skin and the retention within the skin, and increase the efficacy of the compound (Budhiraja et al., 2014; Campos et al., 2012; Visentin et al., 2012). Among different approaches explored so far, microparticles, especially those made of biocompatible polymers, such as chitosan, seem to assure active and safe delivery of this compound (Silva et al., 2014b).

1.3. Microencapsulation

Microencapsulation is a process of encapsulating a material containing an active ingredient (core material) in a shell of a second material (encapsulating/wall material) for the purpose of shielding active ingredients from the surrounding environment permanently or temporarily. This results in small capsules of many useful properties, termed microcapsules or microparticles. Such particles have diameters between one micron and a few millimetres. Particles whose diameter is in the nanometre range are referred to as nanoparticles (Kaur et al., 2013; Jyothi et al., 2010, Benita, 2005). The small size of these particles provides a large surface area that is available for sites of adsorption/desorption, chemical reactions, light scattering, etc. (Arshady, 1999; Gutcho, 1976).

The development of microencapsulation began with the preparation of capsules containing dyes in 1950s by Green and Schleicher. These were incorporated into paper for copying purposes and replaced carbon paper. Nowadays this approach has been widely explored by the pharmaceutical, food, cosmetic, textile, agricultural, veterinary, chemical and biomedical industries (Hammad et al*.,* 2011; Ghosh, 2006). The field with the highest level of microencapsulation applications is the drug sector (68%), followed by food (13%) and cosmetics (8.0%) (Martins et al., 2014). There are numerous possibilities to use microencapsulation as a technique to obtain products with high added value and therefore widespread interest has developed in microencapsulation technology (Martins et al., 2014; Dubey et al., 2009).

Core materials in microcapsules may exist in the form of a solid, liquid or gas. Depending on the application, a wide variety of core materials can be encapsulated, including pigments, fragrances, flavours, enzymes and cells. The size of the core material plays an important role for diffusion, permeability and controlled release. The shell material can be permeable, semi-permeable or impermeable. Compatibility of the core material with the shell is an important criterion for enhancing the efficiency of the microencapsulation, and for this pre-treatment of the core material is often carried out (Estevinho et al., 2013a; Hammad et al., 2011; Ghosh, 2006).

The morphology of the internal structure of a microcapsule (Figure 3) depends largely on the selected shell material and the encapsulation method employed. Microcapsules can be classified as mononuclear, polynuclear or matrix type. Mononuclear microcapsules contain the shell around the core and have a single hollow chamber within the capsule. Microcapsules can also be mononuclear with multiple shells. The polynuclear microcapsules have many cores enclosed within the shell, so they have a number of different sized chambers. The matrix type microparticle has the active ingredients integrated within the matrix of the shell material and distributed homogeneously (Chhotalal et al., 2013; Dubey et al., 2009; Ghosh, 2006).

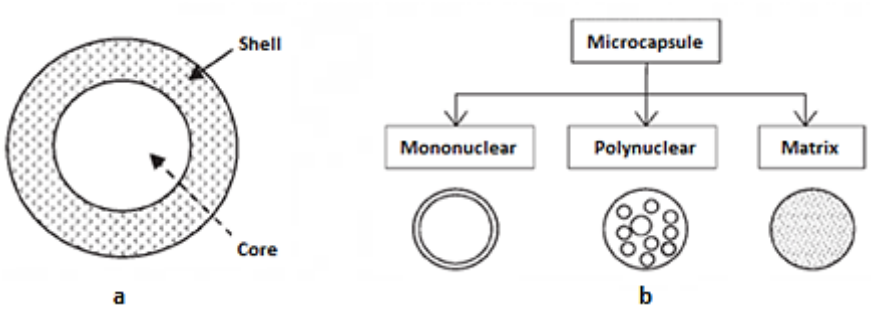


Figure 3. (a) Scheme of a microcapsule. (b) Morphology of microcapsules. (Adapted from Ghosh, 2006)

Microcapsules are usually characterized by parameters such as particle size, zeta potential, encapsulation efficiency and drug loading (Estevinho et al., 2013a). The size and shape of the microparticles can be determined by light or scanning electron microscope (LM or SEM). Encapsulation or association efficiency (content of core material effectively encapsulated) depends on several variables. The retention of the active agent inside the shell is determined by the chemical nature of the core (molecular weight, chemical functionality, polarity and volatility), shell material properties and the encapsulation technique (Selvaraj et al., 2012; Jyothi et al., 2010; Martins et al., 2010; Gander et al., 1995). Microcapsule solvation, density, compressibility index, Hausnner's ratio[2] and angle of repose can also be determined (Hammad et al., 2011).

Microcapsules have a number of interesting advantages, thus microencapsulation technology is used for several purposes: to combine properties of different materials (e.g. organic and inorganic); to protect sensitive, unstable and reactive materials from their environments and prevent the degradation of active compounds (e.g. from reactions like oxidation and dehydration); to protect the immediate environment of the microcapsules from the active components; to increase stability; for controlled, delayed or sustained release; to reduce dosing frequency; for enzyme and microorganism immobilization; to mask undesired properties of the active components (such as odour, taste and activity); for a targeted release of encapsulated materials; for better processability, as it allows to improve solubility, dispersibility and flowability; for a safe and convenient handling of toxic materials; and to separate incompatible components for functional reasons. Some application examples of the use of microencapsulation technology are isolating vitamins from the deteriorating effects of oxygen, retarding evaporation of a volatile core, improving the handling properties of a sticky material or isolating a reactive core from chemical attack (Ramu et al., 2014; Ghosh, 2006; Lopéz et al., 1997).

[2] Index of flow ability of microcapsules.

1.3.1. Microencapsulation techniques

Although a variety of techniques have been reported for microencapsulation, no single process is adaptable to all core materials or product applications. The choice of the most suitable method depends on the application of the microsystem, particle size required, physical and chemical properties of the core and the shell, release mechanism intended, production scale and costs. An appropriate combination of starting materials and synthesis methods can be chosen to produce encapsulated products with a wide variety of compositional and morphological characteristics, and the encapsulation process must be optimized in order to provide a satisfactory outcome, considering the intended application. Microencapsulation techniques can be broadly divided into two main categories, namely chemical and physical, with the latter being further subdivided into physico-chemical and physico-mechanical techniques (Silva et al., 2014a; Estevinho et al., 2013a; Wilson et al., 2007). Table 3 outlines common methods used to encapsulate ingredients and the size of particles they produce. Such methods have been described by Silva et al. (2014a), Dubey et al. (2009), Fairhurst et al. (2008), Wilson et al. (2007) and Ghosh (2006).

Table 3. Usual methods for microencapsulation and respective particle size produced.

Type of Method	Method	Particle size (μm)	References
Chemical process	Emulsion Polymerization	0.5–1000	Fairhurst et al., 2008; Ghosh, 2006; Hirech, 2003
	Suspension Polymerization	0.5–1000	
	Interfacial Polymerization	0.5–1000	
Physico-chemical process	Coacervation/Phase separation	1–1000	Fidalgo et al., 2013; Di Marco et al., 2010; Cocero et al., 2009; Martins et al., 2009; Nguyen-Ngoc et al., 2007; Ghosh, 2006; Freitas et al., 2003
	Solvent evaporation/extraction	0.5–1000	
	Sol-gel encapsulation	2–20	
	Supercritical fluid-assisted microencapsulation	0.5-500	
	Layer-by-layer assembly	0.5–20	
Physico-mechanical process	Spray-drying	1–500	Fernandes et al., 2014, 2013; Shinde et al., 2014; Teodoro et al., 2014; Anwar et al., 2010; Fairhurst et al., 2008; Ghosh, 2006; Senuma et al., 2000
	Spray-cooling	20 – 500	
	Co-extrusion	250–2500	
	Spinning disk	5–1500	
	Fluidized-bed coating	20–1500	
	Melt solidification	5-1000	
	Polymer precipitation	5-1000	

Spray drying is one of the most common methods used for microencapsulation. It is a continuous and single-cycle process that involves the conversion of a material from a fluid state (solution, emulsion or suspension) into a dried particulate form by atomization in a hot gas medium (generally air) (Estevinho et al., 2014b, 2013a, 2013b). The ingredient to be encapsulated is added to the carrier (the ratio of core to carrier can be optimized for each individual ingredient) and the

mixture is homogenized. An emulsifier or cross-linking agent may also be added at this stage. This mixture is then fed into the spray dryer with circulating hot air and atomized, which can be made by different types of atomizers: pneumatic atomizer, pressure nozzle, spinning disk, fluid nozzle and sonic nozzle. The solvent is evaporated by the hot air and the shell material encapsulates the core. Small particles are deposited in the collection vessel where they are collected (Fernandes et al., 2014, 2013; Teodoro et al; 2014; Chávarri, 2012; Wilson et al., 2007). A representation of the spray drying process and an image of the equipment are presented in Figure 4.

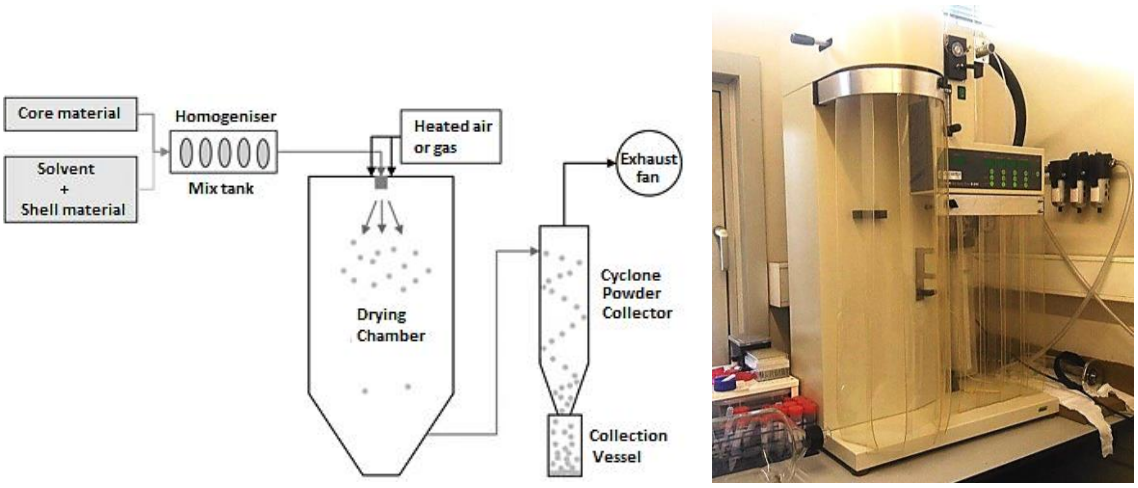


Figure 4. Scheme (Adapted from Chávarri, 2012) and photography of the spray-drying process/equipment.

The properties of the final microparticles depend on the nature of the feeding flow as well as the operating parameters, such as flow rate and inlet temperature. Hot air inlet temperature is typically 150-220 °C and after evaporation the temperature decreases typically to moderate values between 50 and 80 °C. Spray drying can produce, depending on the starting feed material and operating conditions, particles with a size between 1 and 500 µm. Increasing the energy provided to the atomizer decreases the size of the formed particles. For the same energy amount, the size of the particles increases with increasing feed rate. On the other hand, the size of particles also increases with the viscosity and surface tension of the feeding liquid. The microcapsules produced are normally of the polynuclear or matrix type and the mechanisms of release involved are typically controlled by solvents action and diffusion (Estevinho et al., 2014, 2013a; De Vos et al., 2010; Gharsallaoui et al., 2007; Azeredo, 2005).

The shell material, which is hydrated in water, should therefore be soluble in water, have good emulsifying properties, be a good film former, have low viscosity and provide good protection to the encapsulated ingredient. Water-soluble polymers are mainly used as shell materials due to the fact that solvent-borne systems produce unpleasant odours and environmental problems.

Typical shell materials used in spray drying include chitosan, sodium alginate, gum arabic, maltodextrins, modified starch, and mixtures of these. The use of encapsulating agents with low solubility in water implies an increase in the amount of water to be evaporated, and thereby a decrease in the dry matter content and in the amount of active ingredient, resulting in a costly process (Estevinho et al., 2013a; Wilson et al., 2007; Ghosh, 2006).

Spray drying has advantages over other encapsulation methods: large equipment availability, possibility of employing a wide variety of encapsulating agents, easy control of microparticle properties by changing the operational parameters, easy scale-up and large-scale production, low process cost and it is a rapid methodology, reproducible and cost-effective, justifying its use in industrial applications (Estevinho et al., 2014, 2013a, 2013b, 2012; Fernandes et al., 2014; Jafari et al., 2008, 2007). Moreover the process is adaptable to a wide range of feedstock and product specifications, as it can be used with solutions, suspensions, slurries, melts and pastes. Microencapsulation by spray drying is thus a simple and low cost commercial process that has been used to encapsulate mainly flavours (Estevinho et al., 2013a), oils and pigments, but also thermo-sensitive products, such as microorganisms, enzymes and essential oils, due to the very short heat contact time and the high rate of evaporation, resulting in high quality, stable, functional and low moisture content products. Biologically active materials have been successfully spray dried without appreciable activity losses (Estevinho et al., 2014a, 2014b, 2013a, 2013b, 2012; Fernandes et al., 2013).

1.3.2. Microencapsulation in cosmetics

Delivery systems and microcapsules play an important and growing role in the cosmetics and personal care industries nowadays. They offer an ideal and unique carrier system for active ingredients, allowing the controlled and targeted release, isolation and protection of the active compounds, improved stability and efficacy, safe administration, to mask undesirable properties of the active components, such as their odour, and also the improvement of the tactile and visual appearance of a variety of cosmetic and personal care products. In these industries there is a constant look for new and novel delivery systems to safely incorporate many of the new and sensitive active ingredients of the cosmetic products. Finding new delivery systems can allow an easier and simpler use and development of critical emulsion systems, where temperature and water/oil content are very important and sensitive active ingredients must be added in a special and sometimes very difficult way or under very controlled conditions. Microencapsulation has the potential of delivering active ingredients in some difficult systems, e.g. containing glycolic acid, alpha hydroxy acids (AHAs), salicylic acid, high alcohol content or critical water-in-oil or

silicone emulsions. They can be used to deliver active ingredients into the skin, in a safe, targeted, effective and not painful manner, to protect fragrances or volatile compounds from evaporation, to protect compounds such as antioxidants from oxidation, to protect from degradation caused by heat, light and moisture, or to control the release rate (Martins et al., 2010; Pardeike et al., 2009; Soest, 2007; Rosen, 2005). Recent published patents in the area of microencapsulation suggest that both industrial and academic sectors are urging to explore this area, namely in the fields of cosmetics and personal care products (Martins et al., 2014).

Microencapsulation can be used in cosmetic applications for shower and bath gels, lotions and creams, hair products, sunscreens and tanning creams, makeup, perfumes, soaps, tooth pastes and more. Its study and development may help in the improvement of the cosmetic and personal care industries, as microencapsulation technology brings innovation and allows the production of high added value products, in response to human needs and desires (Barel et al., 2001).

1.3.3. Delivery systems for topical application

The skin is the largest and most complex human organ, with at least five different cell types contributing to its structure and representing 16% of total body weight (2 m^2 of surface). The main function of the skin is the protection of the body, which includes physical, chemical and immunological protection, protection against pathogens, and UV radiation and free radical defences. It is also the major participant in thermoregulation, it functions as a sensory organ and it performs endocrine functions. Human skin is comprised of several distinct layers, namely the *stratum corneum* (epidermis), the remaining layers of the epidermis, the dermis and the sub-cutaneous adipose tissue (hypodermis). The epidermis is the outer layer and acts as a protective coating against environmental and external influences. It consists of several layers starting with the *stratum corneum* to the basal cell layer, and is continually being regenerated. The dermis lies beneath the epidermis and is where collagen and elastin are synthesized. It contains the blood vessels, nerves, sweat glands, hair follicles and sebaceous glands. The hypodermis contains the adipose tissue (sub-cutaneous fat) and provides a thermal barrier. The structure of the skin is represented in Figure 5a. The *stratum corneum* is the upper 10–20 µm layer, which is a lipid-rich matrix composed primarily of ceramides, cholesterol and fatty acids that are assembled into a multi-lamellar bilayer structure. The structure of the *stratum corneum* comprises protein rich cells (corneocytes) embedded into the intercellular lipid domains. Corneodesmosomes act to link neighboring corneocytes together and provide structural integrity to the *stratum corneum*. The structure of the *stratum corneum* is represented in Figure 5b (Ammala, 2013; Harding, 2004).

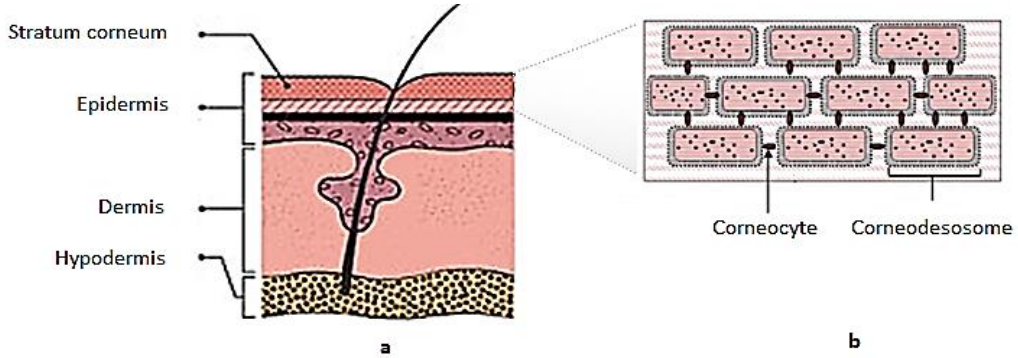


Figure 5. Schematic representation of a) human skin; b) *stratum corneum*. (Adapted from Harding, 2004)

A delivery system is a method of holding, carrying and transporting an active ingredient. It is any type of vehicle that takes an active to a target site. A delivery system can control the release rate of an active ingredient from a formulation at an optimal rate. A number of pathways are possible for the transportation of molecules through the skin. The intercellular route occurs at the interface between cells through the lipid bilayers, following a tortuous permeation pathway. In contrast, transcellular pathways can occur directly through the cells. Transportation via the hair follicles or sweat ducts is also possible (Wiechers, 2008; Flynn, 2002; Moghimi et al., 1999).

Topical application of cosmetic formulations often requires the successful delivery of active ingredients through the skin's barriers to reach the target sites in the body. The main resistance to transdermal transport is in a layer of cells joining the epidermis to the *stratum corneum*, which itself also limits the transport. The *stratum corneum* has a highly impermeable nature, which has remained one of the major challenges in effective transdermal delivery. While the lipophilic *stratum corneum* contains about 13% water, the inner skin epidermis layers become significantly more hydrophilic, containing 50% water, while the dermis contains 70%. It is of extreme importance to understand these properties to design carriers to achieve the desired delivery of cosmetic active substances. It should also be noted that there are wide variations in permeability at different body sites (e.g. face vs. legs vs. palms) which together with factors such as age and external environment can influence the skin's barrier function (Lam et al., 2014; Forster et al., 2009; Wiechers, 2008; Elias, 2004; Harding, 2004; Rein, 1924).

It is generally reported that the transport of molecules through the epidermis is restricted to molecules of low molecular mass (<500 Da) and moderate lipophilicity (partition coefficients log Kow values between 2 and 3), having enough solubility in the lipid domain of the *stratum corneum*, while still having sufficient hydrophilic nature to allow partitioning into the skin inner layers. As some active cosmetic substances are too hydrophilic to pass through the *stratum corneum* or too lipophilic to partition into the epidermis, encapsulation techniques with the right shell materials can overcome this problem by delivering the level of lipophilicity needed for the

desired application. Mathematical models can be used to predict skin permeability, which are generally based on quantitative structure-permeability relationships (QSPR), diffusion mechanisms or combinations of both. At the same time the compound should still have the lipophilic or hydrophilic characteristics that allow its solubilisation in the cosmetic itself and ensure its stability during formulation, storage and application of the product (Ammala, 2013; Forster et al., 2009; Jain et al., 2006; Wiechers, 2005; Barry, 2002; Muller et al., 2002, 2000).

Critical aspects should be considered when delivering a cosmetic active ingredient through the skin, such as the right site of action of the cosmetic ingredient, the right concentration of the components, and the correct application time of the product on the skin. It should be considered the influence of the formulation type, formulation polarity, *stratum corneum* polarity, skin lipid organization and the influence of particle size on skin delivery. The cosmetic delivery should also avoid undesirable transdermal delivery, such as leakage into systemic circulation, and keep the functional molecule in a specific skin layer (Abla et al., 2013; Wiechers, 2008; Fu et al., 2005). Common functional ingredients used in topical application cosmetics are UV filters, antioxidants, moisturizers, skin lightening ingredients, and molecules with anti-aging properties, acting either on the surface of the skin or in specific skin layers (Ammala, 2013; Wiechers, 2008; Elias, 2004).

Several skin delivery systems used in cosmetic products have been reported. Vesicles and niosomes (non-ionic surfactant-based vesicles) are much used as skin delivery vehicles for pharmaceutical and cosmetic purposes. Elastic vesicles showed superior properties in comparison with the conventional, rigid vesicles regarding the function potential and the interactions with the human skin. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) are also much investigated for cutaneous administration. Flexible liposomes are used as delivery systems for topical applications in cosmetics as well. Since the shell material comprises triglycerides and phospholipids, when used in topical delivery, liposomes can facilitate absorption of the active ingredient into the epidermis and they also have the ability to transport active substances into the deeper skin layers. Micellar nanoparticles, which are liposome-like multi-lamellar structures, have also been reported as skin delivery systems. Encapsulation techniques are also much used as skin delivery systems in topical cosmetic products. Encapsulation of actives for cosmetic and pharmaceutical applications have showed a wide range of applications, as the advantages of different encapsulation technologies were made evident. Other delivery systems, such as phytosomes, transferosomes, nanocrystals and cubosomes have also been reported (Chanchal et al., 2008; El Maghraby et al., 2008; Fairhurst et al., 2008; Wiechers, 2008; Kaur et al., 2007; Rosen, 2005; Barry, 2002; Flynn, 2002).

1.3.4. Encapsulating materials

The efficacy of topically applied active ingredients clearly depends on the design of appropriate carriers and the type of vehicle used for their delivery, protection and release. The correct choice of the encapsulating material according to the intended application is essential, as it influences the encapsulation efficiency and the stability of the microparticles. Factors to be considered while selecting a wall material for topical applications include toxicity, biocompatibility, stability, viscosity and mechanical properties, compatibility between the active ingredient and the wall material, release of the active ingredient from the vehicle into the skin, enhancement of active penetration into the *stratum corneum*, intended particle size and microscopic properties of the surface of the microparticles and processing and economic factors. Since most encapsulating materials do not have all the required properties, a common practice involves a combination of wall materials (Silva et al., 2014b; Abla et al., 2013; Estevinho et al., 2013a).

Encapsulating materials can be selected from a wide variety of natural and synthetic polymers. The most commonly used encapsulating materials include polysaccharides (gums, starches, celluloses, alginates, cyclodextrins, maltodextrins, chitosan), proteins (gelatin, casein, soy proteins), lipids (waxes, paraffin, oils), aliphatic polyesters (e.g. poly(lactic acid) – PLA), copolymers of lactic and glycolic acids (e.g. poly(lactic co-glycolic acid) - PLGA) and synthetic polymers (acrylic polymers, polyvinyl alcohol, poly(vinylpyrrolidone)). Inorganic materials, such as silicates, clays and polyphosphates, can also be used as second polymers (Lee et al., 2012; Tarimci, 2011; Cattaneo, 2010; Pawar et al., 2010; Pedro et al., 2009; Wille, 2006; Matsuda et al., 1999).

Biopolymers (natural polymers) and biodegradable polymers are the encapsulating materials with greater interest for applications in the field of skin delivery systems. These materials are natural, non-toxic, non-reactive when in contact with the human tissues and can be broken down or metabolized and removed from the body via normal metabolic pathways, while other compounds can potentially accumulate in body tissues and cause irritation. Their properties are strongly defined by structural characteristics, including composition, molecular weight and nature of the chain end groups, and these polymers can be chemically functionalized to obtain improved properties (Ammala, 2013; Haddadi et al., 2008; Mishra et al., 2008; Stevanovic et al., 2007).

Among numerous entrapping materials used for the encapsulation of pharmaceutical, cosmetic or food active ingredients, chitosan has received much attention for its excellent biocompatibility (Belščak-Cvitanović et al., 2011). Chitosan is a natural and biodegradable linear co-polymer polysaccharide consisting of β-(1–4)-linked D-glucosamine (2-amino-2-deoxy-D-glucose) and N-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose) (Figure 6) (Silva, 2014; Ammala, 2013).

Chitosan is obtained by partial alkaline deacetylation of chitin (a N-acetyl-glucosamine polymer), which is the second most abundant natural polymer in nature. Chitin is synthesized by an enormous number of living organisms and is found predominantly in the exoskeletons shells of crustaceans, insects and other invertebrates (Silva 2014; Estevinho 2013a).

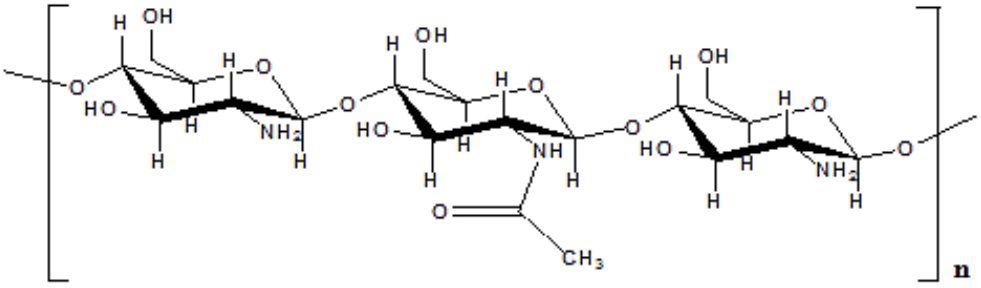


Figure 6. Chemical structure of chitosan.

Chitin is insoluble in aqueous solutions, while chitosan is soluble in acidic solutions due to free amino groups in protonated D-glucosamine units (Silva et al., 2014b; Sashiwa, et al., 2002). Chitosan can be produced with different deacetylation degrees (40–98%) and molecular weights (10–2000 kDa). These two characteristics have a significant role in chitosan properties (e.g. solubility, viscosity) and are extremely important when drug delivery is thought. The higher the deacetylation degree the more chitosan is soluble in water, and the higher the molecular weight more sustained is the release (Estevinho et al., 2013, 2012; Aranaz et al., 2009; Desai et al., 2006).

Chitosan has unique and exceptional biological properties that promote its use as drug carrier. Moreover it is a renewable and inexpensive material with reactive amino functional groups, having the potential to be used in many different applications (Silva, 2014; Estevinho et al., 2013a). Chitosan has been widely reported for use in topical and transdermal delivery systems largely due to its non-toxicity, biocompatibility and biodegradability (Silva, 2014; Ammala, 2013; Aranaz et al., 2009). It has also been reported that chitosan has the ability to enhance permeation across the skin by altering the structure of keratin and that it increases the water content of the *stratum corneum* and cell membrane fluidity. Further, due to its positive charge under slightly acidic conditions, it can depolarize the negatively charged cell membrane and in doing so, it decreases the membrane potential and drives the active component through the skin (Ammala, 2013, Pawar et al., 2010; He et al., 2009). Previous studies also indicated that chitosan possesses various biological activities, such as antitumor and antioxidant effects, anti-cholesterolemic properties and antimicrobial activity against several pathogen and spoilage bacteria (Estevinho e al., 2013a; Fernandez-Saiz et al., 2009; Garcia et al., 2009; Sashiwa et al., 2004; Muzzarelli, 1998; Tokoro et al., 1988).

Chitosan microparticle carriers have an exceptional potential for the delivery of bioactive compounds, since these systems are stable in contact with physiological fluids and barriers, protect the active compounds against adverse conditions and can control the release (Silva et al., 2011). Microparticle properties depend on chitosan molecular weight and deacetylation degree and the mass ratio of chitosan to active ingredient. Water insoluble active ingredients are able to be delivered topically in the form of encapsulated chitosan-based microparticles, making it particularly appealing for topical delivery systems. In addition to topical delivery, chitosan systems have also been reported in oral and transmucosal delivery, and other biomedical, pharmaceutical and food applications, including for the delivery of natural antioxidants (Silva, 2014; Ammala, 2013; Estevinho et al., 2013a; Belščak-Cvitanović, 2011; Harris et al., 2011; Kosaraju et al., 2006).

In spite of the interesting advantages of chitosan, it is insoluble in water and organic solvents, only being soluble in acidic solutions, which frequently limits its application (Silva, 2014). It is possible, however, to modify chitosan structure in order to produce water soluble chitosan, which is easily soluble in neutral aqueous solutions. This increases the range of applicability of this compound, making it very useful as carrier for bioactive ingredients for pharmaceutical, cosmetic and food applications. Chitosan solubility can be improved by chemical modifications involving the introduction of hydrophilic functional groups, such as acetyl, carboxyl, hydroxyl, sulfuryl or phosphoryl groups, and also by a chemical, physical or enzymatic depolymerisation process, which leads to low molecular weight chitosan or oligomers. The localization of the new groups in the molecule of chitosan can change with the type and conditions of the reaction involved and the type of reagents used. Many chitosan derivatives can be produced, such as carboxymethyl chitosan, N-sulfofuryl chitosan, 5- methyl pyrrolidinone chitosan, dicarboxymethyl chitosan, and quaternized chitosan (Estevinho et al., 2014a, 2013a, 2013b; Sashiwa et al., 2004, 2002). Furthermore the chemical modification of chitosan is a powerful tool to control its properties, to control the interaction of the polymer with bioactive compounds, to enhance the loading capability and to tailor the release profile of the particles. Hence, chemically modified chitosan improves its bulk properties for the preparation of delivery systems, which enhances its versatility and applicability in the cosmetics sector. Nevertheless modified chitosan may compromise chitosan biodegradability, as the rate of degradation is generally inversely proportional to the extent of chemical modification (Silva, 2014).

Considering the above factors, chitosan and modified-chitosan were selected as vehicles for this study. The combination of chitosan based particles with a high potent antioxidant compound could be a hope for future delivery of these agents, considering the important effect of oxidative stress in body tissues and oxidative degradation in cosmetic formulations.

1.3.5. Controlled release

Encapsulation should allow the core to be isolated from the external environment until release is desired. Therefore, the release at the appropriate time and place is an extremely important property in the encapsulation process, improving the effectiveness and expanding the applications of compounds of interest. The main factors affecting the release rates are related to interactions between the wall material and the core. Additionally, other factors influence the release, including the ratio between the core and the wall material, particle size, the method of encapsulation and the medium where the release occurs (e.g. pH, ionic strength) (Nikkola, 2014).

The main mechanisms that can be involved in the core release are diffusion, degradation, use of solvents, pH, temperature and pressure. The release of an active ingredient may be based on one or on a combination of release mechanisms and these can be time, site, rate and/or stimulus specific. Diffusion occurs especially when the microcapsule shell is generally intact, and the release rate is governed by the chemical and physical properties of the core and the shell material. Degradation release occurs when enzymes, such as proteases, carbohydrases and lipases, degrade proteins, polysaccharides or lipids, respectively. In contact with a solvent, the shell material can dissolve partly or completely, quickly releasing the core, or start to expand, favouring the release. The pH release can occur when pH changes result in alterations in the shell material solubility, enabling the release of the core. Changes in temperature can promote core release, either by temperature-sensitive release, where materials expand or collapse when a critical temperature is reached, or by fusion-activated release, which involves melting of the shell material due to temperature increase. Pressure release occurs when a pressure is applied to the capsule wall, e.g. the release of cosmetic active ingredients during friction against the skin upon application. Less common release mechanisms include ablation (slow erosion of the shell) and biodegradation. The different release mechanisms provide controlled, sustained or targeted release of the core material, and with a properly designed release system the active ingredient is released at the desired site, time and rate. (Dubey et al., 2009; Nack, 1970).

The study of the controlled release mechanisms helps to overcome both the ineffective utilization and the loss of bioactives during the processing steps. Mathematical modelling of release profiles can help provide a scientific knowledge basis concerning the mass transport mechanisms that are involved in the control of substances release. The release of the active compound in an ideal system may follow zero, half or first order kinetics. The applicability of mathematical models to the controlled release of compounds is increasing in industrial applications (Estevinho et al., 2013a).

CHAPTER 2: STATE OF THE ART

During the last decade there has been a growing interest in the formulation of new cosmetic, food and pharmaceutical products containing natural compounds with antioxidant activity and other beneficial properties. Unfortunately, due to their structure and nature, certain compounds such as polyphenols, in particular rosmarinic acid, are not stable and may interact easily with the matrices in which they are incorporated. Although the formulation of microparticles can offer an efficient solution to overcome such limitations, there are limited studies exploring the potential of encapsulation for rosmarinic acid delivery, and generally they are all very recent (Table 4).

Regarding the cosmetic sector, Budhiraja et al. (2014) explored the topical antibacterial and anti-inflammatory potential of rosmarinic acid, by developing a novel antiacne niosomal gel of RA. The RA-loaded niosomes were prepared by emulsion solvent evaporation[3] and evaluated regarding their antimicrobial activity against *Propionibacterium acnes* and *Staphylococcus aureus*. The niosomes were incorporated in a niosomal gel for sustained delivery to bacteria infected cells and these formulations were studied regarding their antibacterial activity and release profile, where RA content was analysed by UV-Vis spectrometry. RA release from the niosomes was performed in phosphate buffer (PB) pH 5.5 and was found to be 70% after 12 h. RA release from the gel formulation was 50% after 24 h. Results stated that skin permeation and retention was higher for rosmarinic acid niosomal gel as compared to rosmarinic acid gel of equivalent amount of free RA. The results showed that RA and RA-loaded niosomes have powerful inhibitory potential against acne bacteria and can be used as a possible therapy against acne vulgaris.

Kim et al. (2010) explored the potential of rosmarinic acid to prevent skin damage caused by free radicals, thereby reducing the risk of cancer and atherosclerosis. RA-loaded polycaprolactone (PCL) microparticles were prepared by emulsion solvent evaporation for applying RA to cosmetic formulations. Changes in the characteristics of PCL microparticles were investigated for searching the optimum RA loading conditions, where zwitterionic surfactant-PCL particles showed the smallest size distribution and highest association efficiency. The long-term stability of RA was evaluated until 8 weeks in cosmetic formulations by analysing RA content using high performance liquid chromatography (HPLC). It was shown that emulsions containing RA-loaded microparticles exhibited a better long-term stability of RA when compared with those containing only RA.

[3] Encapsulating ingredients and surfactants are dissolved in a water immiscible organic solvent. The aqueous phase containing the active is added to the organic phase and the mixture is sonicated to form an emulsion, followed by the removal of the organic phase using a rotary vacuum evaporator. The active ingredient can also be added to the organic phase depending on solubility. Particles are formed during the evaporation of the organic solvent.

Table 4. Studies on the encapsulation of RA or rich sources thereof for industrial and medical applications.

Encapsulated material	Encapsulating Material	Encapsulation Method	Analytical Method	Application	Results	Reference
Rosmarinic acid	Carnauba wax Surfactant: Tween 80	Hot homogenization	HPLC-DAD	Food	Association efficiency: 99% Particle size: 0.035–0.93 µm The properties were maintained throughout the 28 day of refrigerated storage, and no rosmarinic acid was released by the particles during refrigeration.	Madureira et al., 2015
Rosmarinic acid	Witepsol wax Surfactant: Tween 80	Hot homogenization	HPLC-DAD	Food	Association efficiency: 99% Particle size: 0.27-1.4 µm Stability tests during 28 d in refrigerated conditions showed that the particles maintain their initial properties.	Campos et al., 2014
Rosmarinic acid	Niosomes: sorbitan (surfactant), cholesterol Organic solvent: chloroform	Emulsion solvent evaporation	UV-Vis Spectrometry	Cosmetics	Association efficiency: 65% Particle size: 0.81 µm RA release: 70% at 12 h (in PB pH 5.5), and 50% after 14 h (in a gel formulation) Niosomes of RA were found to increase the skin permeation and retention of RA. Encapsulated RA was effective against *S. aureus* and *P. acne*.	Budhiraja et al., 2014
Rosmarinic acid	Glycerol monostearate Surfactants: Tween 80, soya lecithin Stabilizer: hydrogenated soya phosphatidyl choline	Hot homogenization	UV-Vis Spectrometry	Pharmaceutical	Association efficiency: 62% Particle size: 0.15 µm RA release (in PBS): 97% at 14 h NP-treated rats significantly increased behavioural alterations and attenuated the oxidative stress as compared with the control group.	Bhatt et al., 2014
Rosmarinic acid	Carnuba wax Surfactant: Tween 80	Hot homogenization	HPLC-DAD	Food	Association efficiency: 99% Particle size: 0.035-0.94 µm The smallest, physical and thermally stable SLN were those for 1.5% lipid and 2.0% tween 80.	Campos et al., 2012
Rosmarinic acid	Polycaprolactone (PCL) Organic solvents: methylene chloride and acetone (9:1) Surfactants: polyvinyl alcohol, cetrimonium chloride, sodium laureth sulphate, cocamidopropyl betaine	Emulsion solvent evaporation	HPLC	Cosmetics	Association efficiency: 78% (max) Particle size: 7.0-14 µm Cosmetic cream formulation containing RA-loaded PCL microparticles showed a better long-term stability of the RA compared with those containing only RA.	Kim et al., 2010

Encapsulated material	Encapsulated material	Encapsulated material	Encapsulated material	Encapsulated material	Encapsulated material	Encapsulated material
Rosmarinic acid Sage and Savory extract	Chitosan Sodium tripolyphosphate Acetic acid	Ionic gelation	HPLC-DAD	Pharmaceutical	Association efficiency: 51% Particle size: 0.24 µm Antioxidant activity: 3.65 µmol TE Release (in PBS pH 7.4): ≈ 100% at 60 min All nanoparticles demonstrate to be safe without relevant cytotoxicity	Silva, 2014 Silva et al., 2014b
Rosemary extract	Covalently-based network gels: PEG/β-CD and PEG/glycerol	Reaction of isocyanate end-capped PEG with β-CD or glycerol	HPLC-MS UV-Vis Spectrometry	n.s.	Release (in ethanol and water): 60% at 30min The rosemary extract was readily released from the polymeric gels; consequently the values of antioxidant activity corresponding to these samples were similar to that observed in solution	Ionita el at., 2014
Rosemary extract	Poloxamers Supercritical CO_2	Supercritical anti-solvent	HPLC-UV UV-Vis Spectrometry	n.s.	Product Yield: ≈ 100% (max) Particle size: 1.0-20 µm Release (in PB pH 6.8): ≈ 100% at 60 min	Visentin et al., 2012
Raspberry leaf, Gawthorn, Ground ivy, Yarrow, Nettle and Olive leaf extracts	Sodium alginate, Chitosan Cross-linking solution: calcium chloride, ascorbic acid	Electrostatic extrusion	HPLC-DAD UV-Vis Spectrometry	Food	Encapsulation efficiency: 80-89% Particle size: 10-90 µm Release (in water): ≈ 100% at 60 min Although the antioxidant stability of hydrogel microcapsules was deteriorated during refrigerated storage, which might be attributed to the instability of ascorbic acid, the obtained microbeads delivered significant biological activity and antioxidant potential.	Belščak-Cvitanović et al., 2011

max: maximum obtained; n.s.: not specified; NP: Nanoparticles; SLN: Solid Lipid Nanoparticles; PCL: Polycaprolactone; PEG: Polyethylene glycol; β-CD: β-cyclodextrin; CO_2: carbon dioxide; PB: phosphate buffer; PBS: phosphate buffered saline; HPLC-DAD: high performance liquid chromatography with diode array detection; HPLC-MS: high performance liquid chromatography with mass spectrometry; HPLC-UV: high performance liquid chromatography with ultraviolet detection;

The inclusion of antioxidants, or rich sources thereof (fruits, aromatic herbs, etc.) in foods is also becoming a common procedure in the food industry. Madureira et al. (2015) and Campos et al. (2012, 2014) produced and studied the potential of rosmarinic acid loaded particles for food applications. Campos et al. (2012) prepared RA loaded particles by a hot homogenization[4] method using carnauba wax as lipid and polysorbate 80 as surfactant. The effects of lipid proportion (0.5-1.5% w/v) and surfactant concentration (1.0-3.0% v/v) were evaluated, and it was shown that the smallest, physical and thermally more stable particles were formulations containing 1.0-1.5% lipid and 2.0% surfactant. However the association efficiency was high for all formulations (≈ 99%), which means that the entrapment does not change with the different formulations tested. Campos et al. (2014) prepared RA loaded particles using a hot homogenization method, where witepsol wax was used as lipid and polysorbate 80 as surfactant. Madureira et al. (2015) produced RA loaded particles by hot homogenization using carnauba wax as lipidic matrix and polysorbate 80 as surfactant. The stability of both particles was tested during 28 days stored at refrigeration temperature (5 °C) and the results showed that the particles maintain their initial properties.

Rosmarinic acid has also potential in the pharmaceutical field. Bhatt et al. (2014) investigated the potential use of RA loaded nanoparticles for the effective management of Huntington's disease (a neurodegenerative disease) by nasal delivery. The RA loaded particles were prepared by hot homogenization, in which glycerol monostearate was used as lipid, polysorbate 80 and soya lecithin were used as surfactants and hydrogenated soya phosphatidyl choline as a stabilizer. In vitro RA release profile was studied in phosphate buffered saline (PBS) pH 6.8 by analysing RA content by UV-Vis spectrometry. Nasal delivery of the developed particles followed by the study of behavioural and biochemical parameters in Wistar rats was carried out. Sustained release was observed from the RA-loaded particles and the treatment significantly improved behavioural abnormalities and attenuated the oxidative stress in treated rats. RA release was found to be 97% at 14 h for the optimized formula. Therefore this formula is able to control the release of the drug for a longer period of time, which may ultimately reduce the dosing frequency and may enhance the effectiveness of the therapy. The nasal delivery of the particles produced significant therapeutic action as compared to intravenous application, which confirmed that the developed optimized RA-loaded particles formulation following the non-invasive nose-to-brain RA delivery is a promising therapeutic approach for the effective management of Huntington's disease.

[4] The wall material is warmed to a temperature above the melting point and the active ingredient is added to the melted matrix. The preparation is emulsified into an aqueous phase heated above the melting point of the wall material and homogenised (e.g. through ultrasonication). The resulting solution is left to cool at room temperature.

The potential application of encapsulated natural extracts containing rosmarinic acid, namely extracts of rosemary, has also been investigated, considering the benefits of this compound.

Silva et al. (2014b) investigated the use of chitosan particles as stable and protective vehicles for ocular deliver of rosmarinic acid for medical applications. Chitosan particles incorporating RA and natural extracts were prepared by ionic gelation[5] and characterized in order to evaluate their size, association efficiency, in vitro release, irritancy, toxicity and antioxidant activity. The RA release profile was evaluated in PBS pH 7.4 by HPLC and a fast release was observed in all formulations, reaching almost 100% at 60 min. Encapsulated RA exhibited good antioxidant activity, although lower antioxidant activity was observed in the particles comparing to the free compound. The results also confirmed the absence of particles cytotoxicity and irritancy in the eye.

Visentin et al. (2012) studied the encapsulation of rosemary antioxidants with poloxamers by a supercritical anti-solvent process[6] to protect the antioxidants and to improve its aqueous solubility. Total content of polyphenols was quantified and HPLC analyses were performed to verify the presence of some of the major rosemary antioxidants: carnosic and rosmarinic acid. The release of rosemary polyphenols from the particles was measured in isotonic phosphate buffer solution (pH 6.8) by UV-Vis spectrometry and it was shown that the total polyphenolic content was dissolved from the encapsulated product after 60 min.

Belščak-Cvitanović et al. (2011) studied the potential of encapsulating natural extracts rich in phenolic antioxidants, including rosmarinic acid, in alginate–chitosan systems. The encapsulation of the extracts was performed by electrostatic extrusion[7] and in order to increase the solubility of chitosan, ascorbic acid was used, which additionally increased the antioxidant potential of the encapsulated extracts. The phenolic profile (evaluated by HPLC), mineral content and antioxidant capacity of the original and encapsulated extracts were determined, as well as the release profile in water (total phenolic content analysed by UV-Vis spectrometry) and the size of the obtained particles. A fast release profile was observed, where total release of phenolic compounds occurred at 60 min. Although the antioxidant stability of the microparticles was deteriorated

[5] Chitosan is dissolved in an acidic solution, to which an aqueous cross-linking agent solution is added. Inter- and intra-cross-linking (gelation) of the protonated amine group on chitosan molecules with the negatively charged cross-linking agent anions occurs spontaneously leading to the formation of chitosan particles.

[6] The active ingredient precipitates from droplets of an organic solvent when in contact with a continuous phase of supercritical carbon dioxide (CO_2) (anti-solvent). The organic solvent can be almost completely removed by simply flushing with supercritical CO_2 and dry particles are produced after CO_2 extraction step.

[7] Method based on the use of electrostatic forces to disrupt the liquid filament at the tip of a capillary/needle and to form a charged stream of small droplets.

during one month of refrigerated storage, which was attributed to the degradation of ascorbic acid, the obtained microparticles deliver significant biological activity and antioxidant potential.

Ionita et al. (2014) evaluated the encapsulation and release properties of rosemary extract in covalently-based network gels obtained by the reaction of isocyanate end-capped polyethylene glycol (PEG) with β-cyclodextrin or glycerol. The antioxidant activity of rosemary extract entrapped in polymeric materials was analysed. The results showed that rosemary extract was readily released from the polymeric gels (60% after 30 min) and consequently the values of antioxidant activity corresponding to these samples were similar to that observed in solution.

Although the materials and methods used, the parameters to be analysed and the applications intended in the described studies are significantly different to compare their results, generally all results revealed the potential of rosmarinic acid encapsulation, showing that RA may be stably and efficiently encapsulated and suggesting the need to explore new delivery systems to be applied topically to overcome the limitations of topical RA delivery and effectiveness. However this literature review did not allow to determine the best conditions to be used in the present study considering the application intended: preparation of RA loaded microparticles to be incorporated in cosmetic formulations at an industrial level, since small scale methods were employed, different release mediums were used or at different pH values, or the intended applications were different.

Solvent evaporation and hot homogenization have been the most commonly used methods for rosmarinic acid encapsulation (Madureira et al., 2015; Bhatt et al., 2014; Budhiraja et al., 2014; Campos et al., 2014, 2012; Kim et al., 2010). However the spray drying process has been widely and successfully used as encapsulation method for industrial applications, namely for the encapsulation of natural antioxidants (Bakowska-Barczak et al., 2011; Harris et al., 2011; Sansone et al., 2011; Robert et al., 2010; Kosaraju et al., 2006). Spray drying advantages, as discussed, include large equipment availability, easy control of particle properties, easy large-scale production and low process cost. However this method has never been applied to rosmarinic acid encapsulation and it would be of great interest to evaluate the results. Hence in this work a spray drying process was used to encapsulate RA aiming at an industrial cosmetic application.

Regarding the release profile of microencapsulated rosmarinic acid limited studies are available. Budhiraja et al. (2014) evaluated the release of RA from niosomes in PB pH 5.5 and Bhatt et al. (2014) evaluated RA release from solid lipid particles (SLN) in PBS pH 6.8. The results showed 70% and 97% of release at 12 h and 14 h, respectively. Silva et al. (2014b) studied the release of RA

and natural extracts from chitosan microparticles in PBS pH 7.4 and a fast release was observed reaching almost 100% in 60 min. Belščak-Cvitanović et al. (2011) and Visentin et al. (2012) evaluated the release of rosemary antioxidants from alginate-chitosan and poloxamers microparticles and both results showed a total release of the phenolic compounds after 60 min. The differences observed might be due to the different encapsulating agents, particle sizes, phenolic content, medium in which the particles were dispersed, pH values and temperatures used. Considering the interest of chitosan as encapsulating agent (Chapter 1: Section 1.3.4) and spray drying as encapsulation technique, it would be important to study the release of rosmarinic acid from chitosan microparticles prepared by spray-drying in matrices that simulate formulations for topical applications. Although previous studies report fast release profiles of RA encapsulated in chitosan particles (Silva et al., 2014b), which might not be the optimum behaviour for cosmetic applications, no release studies were performed in oily matrices or solutions balanced with skin pH mimicking cosmetic formulations, and it would be interesting to evaluate the results. Therefore in this work a spray drying process was used to encapsulate RA into chitosan particles and the RA release profile was evaluated in water (pH 5.5) and coconut oil to simulate cosmetic formulations. Coconut oil is a vegetable oil obtained from fresh, mature kernel of the coconut (*Cocos nucifera*) and it comprises various health and nutraceutical benefits, which made it popular in the scientific field. Coconut oil is non-skin irritating and has already been used in emulsions for topical application. The main fatty acid in coconut oil is lauric acid (≈ 45%) which has been reported to have antiviral, antimicrobial and antioxidant activities (Suraweera et al., 2014). Coconut oil is mildly acidic to neutral, being in balance with skin pH. Although the skin pH is 5.5, the pH of cosmetic formulations can range between 5.5 and 7 (Rosen, 2005).

For polyphenols and rosmarinic acid determination HPLC (Madureira et al 2015; Campos et al., 2014, 2012; Silva et al., 2014b; Visentin et al., 2012; Belščak-Cvitanović et al., 2011; Kim et al., 2010) and UV-Vis spectrometry (Bhatt et al., 2014; Budhiraja et al., 2014; Visentin et al., 2012; Belščak-Cvitanović et al., 2011) have been used. As HPLC can separate and identify compounds via retention and is generally more sensitive, accurate, precise and specific, less prone to interferences and works well with trace quantities, it was the analytical method firstly chosen for RA quantification in the present work.

CHAPTER 3: WORK OUTLINE

3.1. Aims of the thesis

This project aims to encapsulate rosmarinic acid (RA) into biopolymer-based microparticles as means to overcome the limitation of RA in cosmetic formulations. The development and characterization of a sustained-release system for rosmarinic acid using a biodegradable polymer and the study of the release profile under topical delivery conditions are intended.

Specific aims include developing and validating an analytical method for RA determination; preparing rosmarinic acid loaded microparticles by the spray-drying technique using different types of chitosan, a natural biodegradable polymer; characterizing the obtained microparticles; studying the controlled release of RA from the particles under topical delivery conditions (water and coconut oil as dispersing mediums, mimicking cosmetic formulations, both balanced with the skin pH) and analysing the antioxidant activity of the encapsulated RA.

3.2. Thesis Organization

This thesis is divided in 5 chapters and respective subchapters. A Background section is firstly presented, where a general introduction of the thesis theme is outlined and some of the reasons why it is a worthwhile question are identified. Chapter 1 is dedicated to a review, presentation and explanation about the theoretical concepts of the subjects and methodologies to be addressed in the thesis. Antioxidants, natural compounds and their relevance in cosmetic applications are discussed. The relevance of rosmarinic acid, which is present in plants such as rosemary (*Rosmarinus officinalis*), for such applications is reported. Microencapsulation, microencapsulation techniques, encapsulating materials and controlled release mechanisms are briefly described, as well as their application to topical delivery cosmetics. Chapter 2 provides a review of the state of the art relevant to this work, explicitly about the encapsulation of rosmarinic acid or rich sources thereof. Chapter 3 presents the aims of the work and the overall organization of the thesis. Chapter 4 describes the materials and methods used for the development, characterization and analysis of biopolymer microparticles encapsulating rosmarinic acid. In chapter 5 the results obtained are presented and discussed. The analytical methods for rosmarinic acid quantification are validated, the microparticles are characterized by size, shape, product yield and association efficiency, the antioxidant potential is assessed and the release profile under topical delivery conditions is studied. The final section presents the main conclusions of the developed work, indicates the limitations emerged and gives suggestions for future research. Additional information to the study is included in the Appendix section.

CHAPTER 4: MATERIALS AND METHODS

4.1. Materials

4.1.1. Standards and Reagents

Rosmarinic acid (Ref. 536954-5G) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Chitosan of medium molecular weight (Ref. 448877-250G), with a viscosity of 200 mPa.s (1%, 25 °C) was obtained from Sigma-Aldrich. Modified chitosan (pharmaceutical grade water soluble chitosan) (Ref. SH20091010) was obtained from China Eastar Group (Dong Chen, China). Modified chitosan (water soluble) was produced by carboxylation and had a deacetylation degree of 96.5% and a viscosity of 5 mPa.s (1%, 25 °C). Methanol and acetonitrile were obtained from VWR International (Radnor, PA, USA). Citric acid was purchased from Merck KGaA (Darmstadt, Germany). Acetic acid was obtained from Carlos Erba (Peypin, France) and ethanol (96%) from Aga (Prior Velho, Portugal). Coconut oil (Ref. C1758-500G) was purchased from Sigma-Aldrich. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), peroxium persulfate and Trolox were obtained from Sigma-Aldrich. Deionised water was obtained in the laboratory using a Merck Millipore Milli-Q water purification equipment (Billerica, MA, USA).

4.1.2. Equipment

All weight measurements were performed using a Mettler Toledo AG245 analytical balance (Columbus, OH, USA). Microencapsulation was performed using a BÜCHI B-290 advanced spray dryer (Flawil, Switzerland) with a standard 0.5 mm nozzle. Instrumental analysis of rosmarinic acid was performed using a Merck Hitachi Elite LaChrom (Tokyo, Japan) high performance liquid chromatograph equipped with a Hitachi L-7100 pump and L-7250 auto sampler, and coupled to a L-7450A diode array detector. HPLC analysis was conducted by using a Purospher® STAR RP-18 endcapped LiChroCART® column (250 mm x 4.0 mm, 5.0 µm) (Merck KGaA), attached to a guard column (4.0 mm x 4.0 mm, 5.0 µm) of the same type. Rosmarinic acid quantification was also performed using a Jasco V-530 UV-Vis Spectrophotometer (Easton, USA), as well as the antioxidant activity assessment. pH measurements were performed using a 900 Multiparameter Water Quality Meter (A & E Lab; Guangzhou, China). Particle morphology was evaluated by Scanning Electron Microscopy, SEM (Fei Quanta 400 FEG ESEM/EDAX Pegasus X4M) and particle size distribution was measured by laser granulometry using a Coulter Counter-LS 230 Particle Size Analyser (Miami, FL, USA).

4.2. Methods

4.2.1. Analytical methodology for rosmarinic acid quantification

Rosmarinic acid was determined and quantified through high performance liquid chromatography with diode array detection (HPLC-DAD) and through UV-Vis spectrometry. HPLC was the methodology firstly chosen for RA quantification, but due to column blocking and damage caused by the microparticles, a UV-Vis spectrometry method was developed.

Analytical Standard Preparation

A stock standard solution of 10 $g.L^{-1}$ of rosmarinic acid was prepared in methanol by accurately weighting 100 mg of RA reference standard using an analytical balance (Mettler Toldedo) into a 10 mL volumetric flask. The stock solution was sealed with parafilm, wrapped in aluminium foil to protect from light, stabilized for 24 hours at 4 °C to ensure homogenization and stored at -20 °C. An intermediate standard solution of 1 $g.L^{-1}$ of rosmarinic acid was prepared from the stock solution by dilution in distilled water into a 10 mL volumetric flask. The intermediate solution was stabilized for 2 hours at 4 °C before further standard preparation.

For HPLC, six working standard solutions were prepared by dilution of the intermediate solution in filtered distilled water: 10, 15, 20, 30, 40 and 50 $mg.L^{-1}$; and four working standards were obtained by dilution of the 50 $mg.L^{-1}$ standard: 0.50, 1.0, 2.0 and 5.0 $mg.L^{-1}$. Distilled water was filtered through 0.45 µm Nylon 66 filter membranes (Sigma-Aldrich). For UV-Vis spectrometry five working standard solutions were prepared by dilution of the intermediate solution in distilled water: 5.0, 8.0, 10, 15, 20 $mg.L^{-1}$; and three working standards were obtained by dilution of the 20 $mg.L^{-1}$ standard: 0.50, 1.0 and 2.0 $mg.L^{-1}$.All measurements were performed using 500 µL and 50 µL SGE Syringes (Melbourne, Australia). Standards were stabilized for 2 hours before analysis.

High Performance Liquid Chromatography

All HPLC runs were performed using a Merck-Hitachi Elite LaChrom high performance liquid chromatograph equipped with a Hitachi L-7100 pump and a L-7250 auto sampler, and coupled to a L-7450A diode array detector. Results were acquired and processed with D-7000 Multi-HSM Manager Software for data acquisition (Merck-Hitachi). HPLC analysis was conducted by using a Purospher® STAR RP-18 endcapped LiChroCART® column, 250 mm in length and 4.0 mm in diameter, with 5.0 µm particle size (Merck KGaA), attached to a guard column (4.0 mm x 4.0 mm, 5.0 µm) of the same type. The mobile phase, at a flow rate of 0.8 $mL.min^{-1}$, consisted of 75% 10 mM citric acid solution in distilled water (acidity adjusted to pH 2.6) and 25% acetonitrile. Distilled water was filtered through 0.45 µm filter (Nylon 66, Sigma-Aldrich) and both phases were

degassed, as well as all washing solutions. Rosmarinic acid chromatographic analysis was performed isocratically. The injection volume was 100 µL and the analysis was performed at room temperature (≈ 20 °C). Detection was performed by measuring the absorption at 330 nm.

The development of the HPLC method was initially based on the method for determination of chlorogenic acids by Tfouni et al. (2014) but the conditions were investigated to provide a simple procedure with the best peak resolution regarding symmetry and tailing, appropriate run time and cost-effective analysis. Modifications to the original method include alteration of the mobile phase and chromatographic column. Five citric acid:acetonitrile ratios (90:10, 80:20, 75:25, 60:40 and 50:50) and two flow rates (1.0 and 0.80 $mL.m^{-1}$) were evaluated regarding the mobile phase.

Ten external standards of rosmarinic acid were run (0.50, 1.0, 2.0, 5.0, 10, 15, 20, 30, 40 and 50 $mg.L^{-1}$) and the calibration curve was constructed, in the chromatographic conditions established above. Retention time was used to identify RA and the total peak area was used for quantification.

UV-Vis Spectrometry

All spectrometry analyses were performed using a Jasco V-530 UV-Vis Spectrophotometer. In order to determine the best detection wavelength for analysis, in which the maximum absorption occurs, rosmarinic acid (0.50 $mg.L^{-1}$ in water and coconut oil) was scanned between wavelengths 200 to 600 nm and the absorption spectra was obtained. Considering the maximum absorption wavelength from the spectra, detection was performed at 327 nm in all spectrometry analyses.

Eight external standards of rosmarinic acid were analysed (0.50, 1.0, 2.0, 5.0, 8.0, 10, 15, 20 $mg.L^{-1}$) and two calibration curves were constructed: in distilled water and in coconut oil. The value of absorption at 327 nm was used to quantify rosmarinic acid.

Analytical Methods Validation

The HPLC and the UV-Vis spectrometry methods were validated, in order to demonstrate that these methods are suitable for quantitative determination of RA and ensure the reliability of the results. Validation performance parameters: quantification parameters (linearity, sensitivity and limits of detection and quantification) and reliability parameters (repeatability and intermediate precision) were determined.

The linearity is the ability of a method to demonstrate that the results obtained are directly proportional to the concentration of the analyte in the sample within a specific range. The results of the standard solutions analysed (in duplicate) by each method were processed statistically to

determine the calibration curve equation (least squares method) and the correlation coefficient R^2, using the Microsoft Excel 2013 software. The calibration curves were constructed plotting the total peak areas, for HPLC, and the values of absorption, for UV-Vis spectrometry, in the vertical axis and rosmarinic acid concentrations in the horizontal axis. The linearity should be evaluated from the analysis of at least 5 standard concentrations in a range factor superior to 10, and the correlation coefficient should be at least 0.99. The sensitivity of the method is expressed as the slope of the calibration curve. The validation of a calibration curve assumes that the following conditions are met:

- analysis of at least 5 different standard concentrations
- linearity range in a factor superior to 10
- $R^2 \geq 0.99$
  $\frac{s_a}{a} \leq 5\%$
- $\mathrm{b} - ts_b < 0 < \mathrm{b} + ts_b$

where a is the regression slope, b the intercept of the regression and s_a and s_b the respective standard deviations.

The limit of detection (LOD) is the lowest amount of analyte in the sample that can be detected by the methodology being validated, while the limit of quantification (LOQ) is the lowest amount of analyte in a sample that can be determined with acceptable accuracy and precision. For HPLC LOD and LOQ were calculated at signal/noise ratio of 3 (S/N=3) and 10 (S/N=10), respectively, for the lowest standard concentration, using the following expression (Eq. 1):

$$LOD, LOQ = C \times \frac{S}{N} \times \frac{N}{H} \quad \text{(Eq.1)}$$

where S/N is the signal/noise ratio (for LOD, S/N=3; and for LOQ, S/N=10), C is the sample concentration, N the noise value when the blank is analysed, and H the value of the signal when the sample is analysed. For UV-Vis spectrometry LOD and LOQ were determined based on the standard deviation of the response and on the slope of the calibration curve, using the following expressions (Eq. 2 and Eq. 3), respectively:

$$LOD = \frac{3 \times s_b}{a} \quad \text{(Eq. 2)}$$

$$LOQ = \frac{10 \times s_b}{a} \quad \text{(Eq. 3)}$$

The precision describes the closeness of the results obtained in a series of measurements of the same sample. Precision is further subdivided into intra-day precision or repeatability, which assesses precision within a short period of time with the same analyst and instrumentation, and inter-day precision or intermediate precision, which measures the precision with time (often

days) and may involve different analysts, equipment, and reagents. Precision was assessed by testing the repeatability of three different standard solutions (low, medium and high concentration: 2.0, 15 and 40 $mg.L^{-1}$ for HPLC, and 1.0, 8.0 and 15 $mg.L^{-1}$ for UV-Vis spectrometry) six times, and by intermediate precision, analysing the same three standard solutions three times on different days. Precision was expressed as coefficient of variation (CV%), and these values should be less than 5%.

4.2.2. Preparation of RA-loaded microparticles

Modified (water-soluble) chitosan solution was prepared with the concentration of 10 $g.L^{-1}$ (1% w/v) by accurately weighting 1 g of modified chitosan (Mettler Toledo) into 100 mL a volumetric flask. Chitosan solution was prepared with the concentration of 10 $g.L^{-1}$ (1% w/v) by accurately weighting 1 g of chitosan (Mettler Toledo) into 100 mL of 1% v/v acetic acid aqueous solution. A solution of 1 $g.L^{-1}$ (0.1% w/v) of rosmarinic acid was prepared by accurately weighting 50 mg of RA (Mettler Toledo) into a 50 mL volumetric flask. All the solutions were prepared with deionised water and at room temperature. Encapsulation agent solutions were prepared on the day before encapsulation and kept overnight at room temperature under stirring (540 rpm) to ensure full saturation of the polymer molecules. Rosmarinic acid solution, also prepared on the day before encapsulation, was sealed with parafilm, wrapped in aluminium foil to protect from light and stabilized for 24 hours at 4 °C. 20 mL of rosmarinic acid solution was added and mixed with each encapsulation agent solution at a constant agitation speed of 540 rpm during 30 min at room temperature before encapsulation by spray-drying. Rosmarinic acid final concentration in the solutions was 2% (w/v). The mass ratio of rosmarinic acid to wall material was 1:20 (w/w).

Microencapsulation was performed using a BÜCHI B-290 advanced spray dryer (Flawil, Switzerland) with a standard 0.5 mm nozzle. The mixtures were feed into the spray dryer at a flow rate of 4 $mL.min^{-1}$ (15%) and an inlet temperature of 115 °C. Air pressure, aspiration rate and nozzle cleaner were set to 6.0 bar, 100% and 3, respectively. The outlet temperature is a consequence of the other experimental conditions and of the solution properties and was around 60 °C. The operating conditions were previously optimized by Estevinho et al. (2014b) and were used accordingly. The dried powders were collected and stored in falcon tubes, wrapped in aluminium foil, and stored at 4 °C while waiting for further analysis.

4.2.3. Characterization of RA-loaded microparticles

Product yield

Product yield (%) was measured for each microencapsulation experiment and was expressed as the ratio of the mass of powder obtained at the spray-dryer output and the solid content of the initial infeed solution (mixture of the encapsulation agent solution and rosmarinic acid solution).

Association efficiency

Association efficiency (AE) was evaluated considering the amount of rosmarinic acid associated with the particles. The AE was calculated by the difference between the total rosmarinic acid used to prepare the particles, and the amount of residual rosmarinic acid in the solution right after dispersion of the particles in water. AE of rosmarinic acid in rosmarinic acid microparticles was obtained according to the following equation (Eq. 4):

$$\mathrm{AE}\% = \frac{\text{Total amount of rosmarinic acid} - \text{Free amount of rosmarinic acid}}{\text{Total amount of rosmarinic acid}} \times 100 \qquad \text{(Eq. 4)}$$

Particle morphology and particle size distribution

Particle morphology was evaluated by Scanning Electron Microscopy, SEM (Fei Quanta 400 FEG ESEM/EDAX Pegasus X4M). Samples of microparticles were gold coated under vacuum in a Jeol JFC 100 apparatus at Centro de Materiais da Universidade do Porto (CEMUP) and SEM analysis was performed at 15 kV with magnification of 100–30 000 x, for surface structure observation.

Particle size distribution was measured by laser granulometry using a Coulter Counter-LS 230 Particle Size Analyser. A small powder sample was suspended in ethanol and ultrasound-irradiated for 10 s to avoid particle agglomeration. The particles were characterized by volume and number distribution using Laser Diffraction and Polarization Intensity Differential Scattering. The results were obtained as an average of two 60 s runs.

4.2.4. Controlled Release study

The study of rosmarinic acid release profiles from the obtained microparticles was performed in two different mediums: distilled water (pH 5.5) and coconut oil, to simulate vehicles for incorporation of microparticles for skin delivery in cosmetic formulations. 4 mg of the microparticles obtained were suspended in 4 mL of each medium and stirred at a constant agitation speed of 60 rpm at room temperature for water and at 28 ˚C for coconut oil to ensure a liquid state. Separate samples were produced and analysed at defined time intervals: 0.0 min, 1.0 min, 2.0 min, 5.0 min, 10 min, 20 min, 30 min, 45 min, 1.0 h, 2.0 h, 4.0 h, 6.0 h and 24 h. The release analysis was accomplished by evaluating the amount of rosmarinic acid released from the particles over the specified time using the HPLC-DAD and UV-Vis spectrometry methods validated

previously (Section 4.2.1). Duplicate samples were analysed at each time. Before HPLC injections, samples were filtered first through a 0.45 µm filter (VWR International) and later through a 0.20 µm filter (VWR International) using 3 mL syringes to ensure particle retention.

4.2.5. Antioxidant activity Assessment

The assay used to estimate the antioxidant capacity of the encapsulated rosmarinic acid was the ABTS radical scavenging assay. A stock solution included 7.4 mM ABTS (Sigma-Aldrich) aqueous solution and 2.6 mM potassium persulfate (Sigma-Aldrich) aqueous solution. The working solution was prepared by mixing equal quantities of the two stock solutions, and the mixture was allowed to react for 12 h at room temperature in the dark. The resulting solution was diluted by mixing 1 mL with 60 mL of methanol to obtain an absorbance of 1.10 ± 0.02 AU at 734 nm. The samples (150 µL) were then mixed with the prepared solution (2850 µL) (1:20 v/v) and allowed to react for 2 h in the dark. Samples consisted of RA loaded chitosan and modified chitosan microparticles at maximum release (after 4 h in aqueous solution) and RA reference standard free in solution in the concentration expected at maximum release, considering controlled release study results (19.6 $mg.L^{-1}$). The absorbance was taken at 734 nm (Jasco V-530 UV-Vis Spectrophotometer). The reactivity of the antioxidants tested was compared to that of Trolox (Sigma-Aldrich), a synthetic analogue of vitamin E commonly used as antioxidant reference. The standard curve was linear between 25 and 400 µM Trolox and the results were expressed in µM Trolox equivalents (TE) (Thaipong et al., 2006).

4.2.6. Quality assurance and control

Rosmarinic acid is a powerful but sensitive antioxidant. Methanolic stock solution of RA was preserved in the freezer (-20 °C) and protected from light to avoid degradation. Aqueous intermediate solution and individual standards were prepared and analysed freshly, and were not stored longer than 24 h. RA microparticles were wrapped in aluminium foil to protect from light and stored at 4 °C.

4.2.7. Waste treatment

The waste generated in this work consisted of aqueous solutions containing rosmarinic acid, chitosan, modified chitosan and coconut oil. A mixture of methanol, ABTS and potassium persulfate was obtained during the antioxidant activity assessment. HPLC analysis generated solutions containing acetonitrile and traces of rosmarinic acid and citric acid. All these residues were collected in closed containers, properly labelled for further treatment by the Environmental Management System of FEUP – EcoFEUP.

CHAPTER 5: RESULTS AND DISCUSSION

To study the release profile of RA from the microparticles, analytical methods for RA quantitative determination had to be developed. In the first phase of this work the development and validation of the chromatographic (HPLC) method for rosmarinic acid determination was carried out. Given the obtained results of the controlled release study by chromatographic analysis, UV spectrometry methods were developed and validated. The results obtained for the validation of the mentioned analytical methods are gathered in subchapter 5.1: Analytical Methods Validation.

After rosmarinic acid encapsulation into chitosan and modified-chitosan microparticles, these particles were characterized regarding particle size, shape and size distribution. The product yield of the encapsulation process and the association efficiency were also determined. These results are presented in subchapter 5.2: Rosmarinic acid encapsulation and characterization of the microparticles.

The controlled release study of rosmarinic acid from the obtained particles was planned to best simulate topical delivery conditions and to study the consequent biopolymer-based microparticles behaviour over a spaced period of time, until 24 h. The study of the release profile was performed in water and coconut oil, to simulate vehicles for incorporation of microparticles for skin delivery in cosmetic formulations. The instrumental analysis for rosmarinic acid quantification was performed by HPLC, and later by UV-Vis spectrometry, considering instrumental problems of the chromatographic method. The results are gathered in subchapter 5.3: Controlled Release. Finally the antioxidant activity of the encapsulated rosmarinic acid as compared to RA free in solution and Trolox (used as antioxidant reference) was assessed. The results are presented in subchapter 5.4: Antioxidant activity.

5.1. Analytical Methods Validation

Considering the need to evaluate the percentage of RA released from the microparticles during the controlled release studies, it was necessary to develop robust analytical methods for RA quantitative determination. As discussed in Chapter 2: State of the Art, both HPLC and UV-Vis spectrometry methods have been used for rosmarinic acid quantification. As HPLC allows the separation, identification and quantification of compounds, is less prone to interferences and is generally a very sensitive, precise and specific method, it was the analytical method firstly chosen for RA quantitative determination in this work

5.1.1. HPLC

The development of the HPLC method for RA quantification was optimized to provide a simple procedure with the best peak resolution regarding symmetry and tailing, appropriate run time and cost-effective analysis. Different citric acid:acetonitrile ratios and flow rates were evaluated regarding the mobile phase. Mobile phase at a flow rate of 0.8 $mL.min^{-1}$ and consisting of 75% 10 mM citric acid aqueous solution (pH 2.6) and 25% acetonitrile was shown to be optimum. A chromatogram obtained for these optimum conditions is shown in Figure 7. The retention time of rosmarinic acid in these conditions was 11.02 ± 0.32 min.

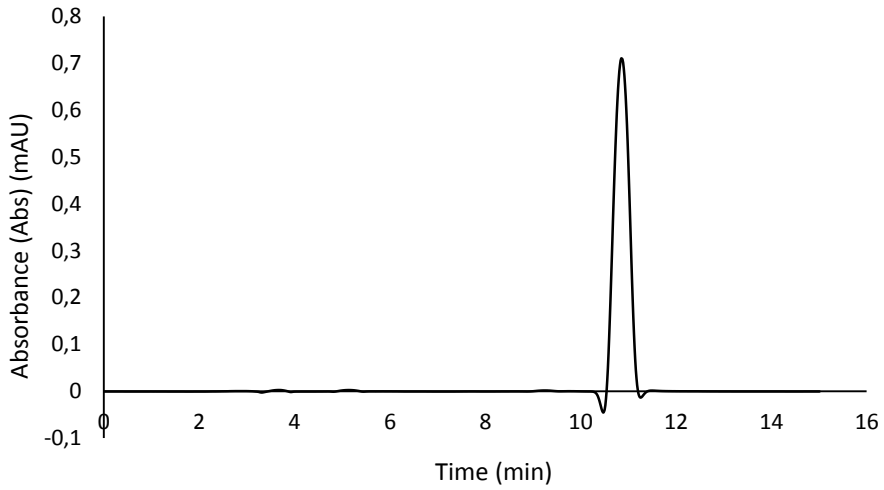


Figure 7. Chromatogram of rosmarinic acid (20 $mg.L^{-1}$) analysed by HPLC-DAD at 330 nm.

After the preliminary tests to optimize the chromatographic conditions, the analytical method was validated to ensure that the method accurately quantifies RA and produces valid and reliable results. Ten external standards of rosmarinic acid in a range of concentrations between 0.50 and 50 $mg.L^{-1}$ were injected in duplicate. The results allowed the construction of the calibration curve, which is presented in Figure A2, Appendix B.1.

Validation performance parameters, including quantification parameters (linearity, sensitivity and limits of detection and quantification) and reliability parameters (repeatability and intermediate precision) were determined.

Regarding the quantification parameters, the results are presented in Table 5. Concerning the linear response of the detector, the regression curve was linear in the studied concentration range. LOD and LOQ were satisfactory low, as their values were less than the lowest concentration of the calibration curve.

Table 5. Quantification parameters of the HPLC method for rosmarinic acid quantification.

Linearity Range	0.50-50 $mg.L^{-1}$	**Correlation coefficient (R^2)**	0.9995
Regression slope Sensitivity	204568 $L.mAU.mg^{-1}$	**LOD**	0.02 $mg.L^{-1}$
Regression intercept	-3642 mAU	**LOQ**	0.08 $mg.L^{-1}$

The linearity conditions for the validation of the calibration curve are presented in Table 6. The results obtained were very satisfactory since the linearity requirements were all met.

Table 6. Linearity conditions for the validation of the HPLC calibration curve.

Linearity parameter	Required conditions	Results obtained
Number of standards	≥ 5	10
Linearity factor range	≥ 10	100
Correlation coefficient (R^2)	≥ 0.9990	0.9995
Slope error	$\frac{s_a}{a} \leq 5\%$	0.76%
Intercept confidence interval	$\mathrm{b} - ts_b < 0 < \mathrm{b} + ts_b$	-40742 < 0 < 33457

Reliability results are presented in Table 7, and in detail in Appendix B.1. Precision, expressed as the coefficient of variation (CV%), was assessed by testing the repeatability (intra-day precision) and the intermediate precision (inter-day precision) at different concentrations within the working range: low (2.0 $mg.L^{-1}$), medium (15 $mg.L^{-1}$) and high (40 $mg.L^{-1}$) standard concentrations.

Table 7. Reliability parameters of the HPLC method for RA quantification.

RA standard concentration ($mg.L^{-1}$)	Precision (CV%)	
	Repeatability	Intermediate precision
2.0	1.9	0.6
15	2.2	0.7
40	2.2	1.0

From the results obtained it can be concluded that the detector used showed no significant response variations and that the precision was satisfactory, as all repeatability and intermediate precision results showed coefficients of variation values lower than 5%.

5.1.2. UV-Vis Spectrometry

Due to the mentioned instrumental problems of the HPLC method, a UV-Vis spectrometry method was developed. Firstly the absorption spectra of rosmarinic acid was obtained between wavelengths 200 to 600 nm, for both controlled release mediums (water and coconut oil) in order to determine the wavelength of maximum absorption in each medium (Figure 8). In water the results showed a maximum absorption at 327 nm. In the case of coconut oil, the band of maximum absorption was not clear, so detection was performed by measuring the absorption at

327 nm for all spectrometry analyses, since this value, obtained for the aqueous solution, is close to the UV maximum absorption wavelength of RA reported in the literature.

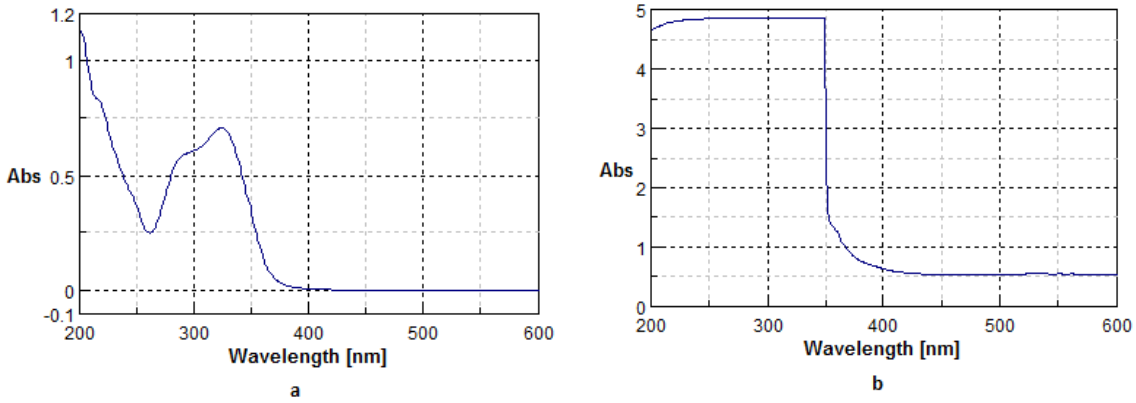


Figure 8. Absorption spectra of RA (0.50 mg.L^{-1}) between 200 and 600 nm for a) water and b) coconut oil.

The UV-Vis Spectrometry analytical methods were also validated, in order to demonstrate that they are suitable for quantitative determination of RA and ensure the reliability of the results. Eight external standards of rosmarinic acid in a range of concentrations between 0.50 and 20 mg.L^{-1} were analysed in duplicate. The results allowed the construction of the calibration curves, which are presented in Figure A3 (a and b), Appendix B.2.

Validation performance parameters: quantification parameters (linearity, sensitivity and limits of detection and quantification) and reliability parameters (repeatability and intermediate precision) were determined.

The results of the quantification parameters of the UV-Vis spectrometry method for RA quantification in water and coconut oil are presented in Table 8. Regarding the response, the regression curve was linear in the studied concentration range. LOD and LOQ were satisfactory low, as LOD values were less than the lowest concentration of the calibration curve, and LOQ values were lower than the concentrations analysed in the controlled release study.

Table 8. Quantification parameters of the UV-Vis spectrophotometer for RA quantification in water and coconut oil.

	Water	Coconut oil
Linearity Range	0.50-20 mg.L^{-1}	0.50-20 mg.L^{-1}
Regression slope / Sensitivity	0.048 L.AU.mg^{-1}	0.054 L.AU.mg^{-1}
Regression intercept	0.0057 AU	0.0047 AU
Correlation coefficient (R^2)	0.9991	0.9996
LOD	0.38 mg.L^{-1}	0.25 mg.L^{-1}
LOQ	1.27 mg.L^{-1}	0.83 mg.L^{-1}

The linearity conditions for the validation of the calibration curves are presented in Table 9. The results obtained were very satisfactory since the linearity requirements were all met.

Table 9. Linearity conditions for the validation of the UV-Vis spectrometry calibration curves.

Linearity parameter	Required conditions	Results obtained	
		Water	Coconut oil
Number of standards	≥ 5	8	8
Linearity factor range	≥ 10	40	40
Correlation coefficient (R^2)	≥ 0.999	0.9991	0.9996
Slope error	$\frac{s_a}{a} \leq 5\%$	1.26%	0.82%
Intercept confidence interval	$b - ts_b < 0 < b + ts_b$	-0.0004 < 0 < 0.0118	-0.0002 < 0 < 0.0092

Reliability results are presented in Table 10, and in detail in Appendix B.2. Precision, expressed as the coefficient of variation (CV%), was assessed by testing the repeatability (intra-day precision) and the intermediate precision (inter-day precision) at different concentrations within the working range: low (1.0 $mg.L^{-1}$), medium (8.0 $mg.L^{-1}$) and high (15 $mg.L^{-1}$) standard concentrations.

Table 10. Reliability parameters of the UV-Vis spectrophotometer for RA quantification in water and coconut oil.

RA standard concentration ($mg.L^{-1}$)	Precision (CV%)			
	Repeatability		Intermediate precision	
	Water	Coconut oil	Water	Coconut oil
1.0	4.9	5.0	1.5	2.7
8.0	3.4	4.0	1.8	1.7
15	0.5	1.1	0.4	0.5

From the results obtained it can be concluded that there was no significant response variations and that the precision was satisfactory, as all repeatability and intermediate precision results showed coefficients of variation values lower than 5%. The precision results in water were slightly better than those in coconut oil, possibly due to better homogenisation of the solution.

5.2. Rosmarinic acid encapsulation and characterization of the microparticles

5.2.1. Product yield

Rosmarinic acid was encapsulated into chitosan and modified chitosan microparticles by a spray drying process. The product yield (quantity of powder recovered in relation to the amount of raw materials used) was 42.6% for chitosan and 39.8% for modified chitosan, which are satisfactory values for the technique applied and the scale that was used. The spray drying method has successfully been used by other authors for the encapsulation of compounds, such as natural antioxidants, with chitosan and other encapsulating agents for industrial applications with

product yields ranging from 24.1% to 82.3% (Bakowska-Barczak et al., 2011; Sansone et al., 2011; Desai et al., 2005). Bakowska-Barczak et al. (2011) showed that the product yield of the spray drying process depends mainly on the type of encapsulating agent used and the inlet temperature employed. On the other hand, if small quantities of raw materials are used when compared to the scale of the spray dryer, which is the case in the present study, several losses will occur throughout the equipment, leading to a lower product yield. Furthermore, the particles produced are of very small size, and there might be some particle aspiration by the vacuum system.

5.2.2. Association efficiency

The RA association efficiency (amount of rosmarinic acid associated with the particles) was 92.6% for chitosan microparticles and 59.6% for modified chitosan microparticles. The pH of chitosan microparticles formation was lower than the one of modified chitosan, as acetic acid was added in order to protonate chitosan amino groups and make chitosan more soluble in water. The more acidic pH favours the interaction of rosmarinic acid and chitosan, since at lower pH values the protonation of chitosan amino groups is higher and reversible ionic interactions between the carboxylate group (COO^-) of rosmarinic acid (pKa = 3.57) and protonated amino groups (NH_3^+) of chitosan (pKa = 6.5) may occur (Figure 9). Therefore the preparation of chitosan microparticles in acidic pH led to the entrapment of higher amounts of rosmarinic acid, resulting in a higher association efficiency. Studies that encapsulated phenolic compounds in chitosan particles obtained association efficiencies in the range of 27% to 90%. (Dudhania et al., 2010; Kosaraju et al., 2006). The entrapment efficiency of chitosan particles can vary from about 20% to 100%, depending mainly on the chitosan molecular weight, the pH upon particle formation and the use of cross-linking agents (Peres et al., 2011).

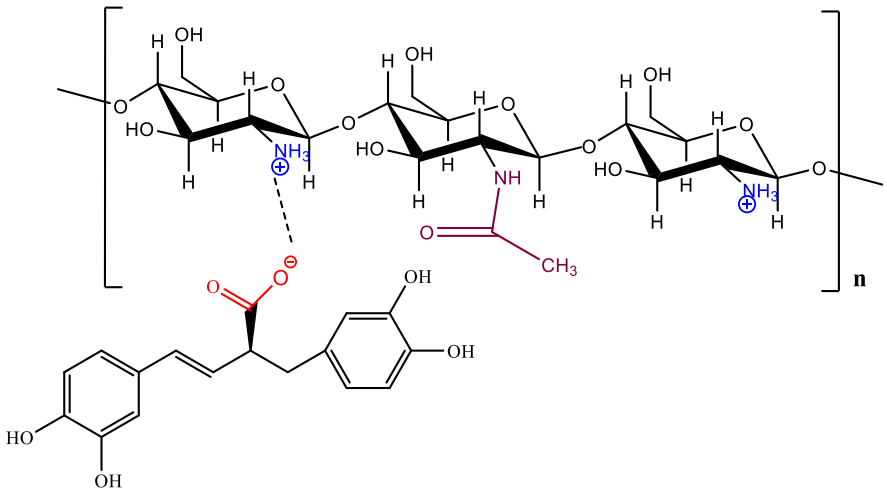


Figure 9. Possible reversible ionic interactions between COO^- group of RA and NH_3^+ of chitosan.

5.2.3. Particle morphology and particle size distribution

The prepared RA-loaded microparticles were analysed by scanning electron microscopy (SEM) for particle morphology analysis and laser granulometry to determine particle size distribution. Figure 10 shows SEM images of RA loaded chitosan and modified chitosan microparticles. The microstructural analysis confirmed a population of smooth, regular and spherical particles for modified chitosan. Chitosan particles showed to be regular and spherical in shape but have a more rough surface morphology, where indentations appeared on the surface of the microparticles. Other authors have reported the presence of wrinkles and indentations on the surface of spray dried chitosan microparticles (Harris et al., 2011; Kosaraju et al., 2006; Desai et al., 2005). Some agglomeration can also be observed from SEM image results. Similar morphology results were obtained by Silva (2004) for chitosan encapsulating RA and by other studies that encapsulated phenolic compounds in chitosan particles (Harris et al. 2011; Sansone et al., 2011; Kosaraju et al., 2006).

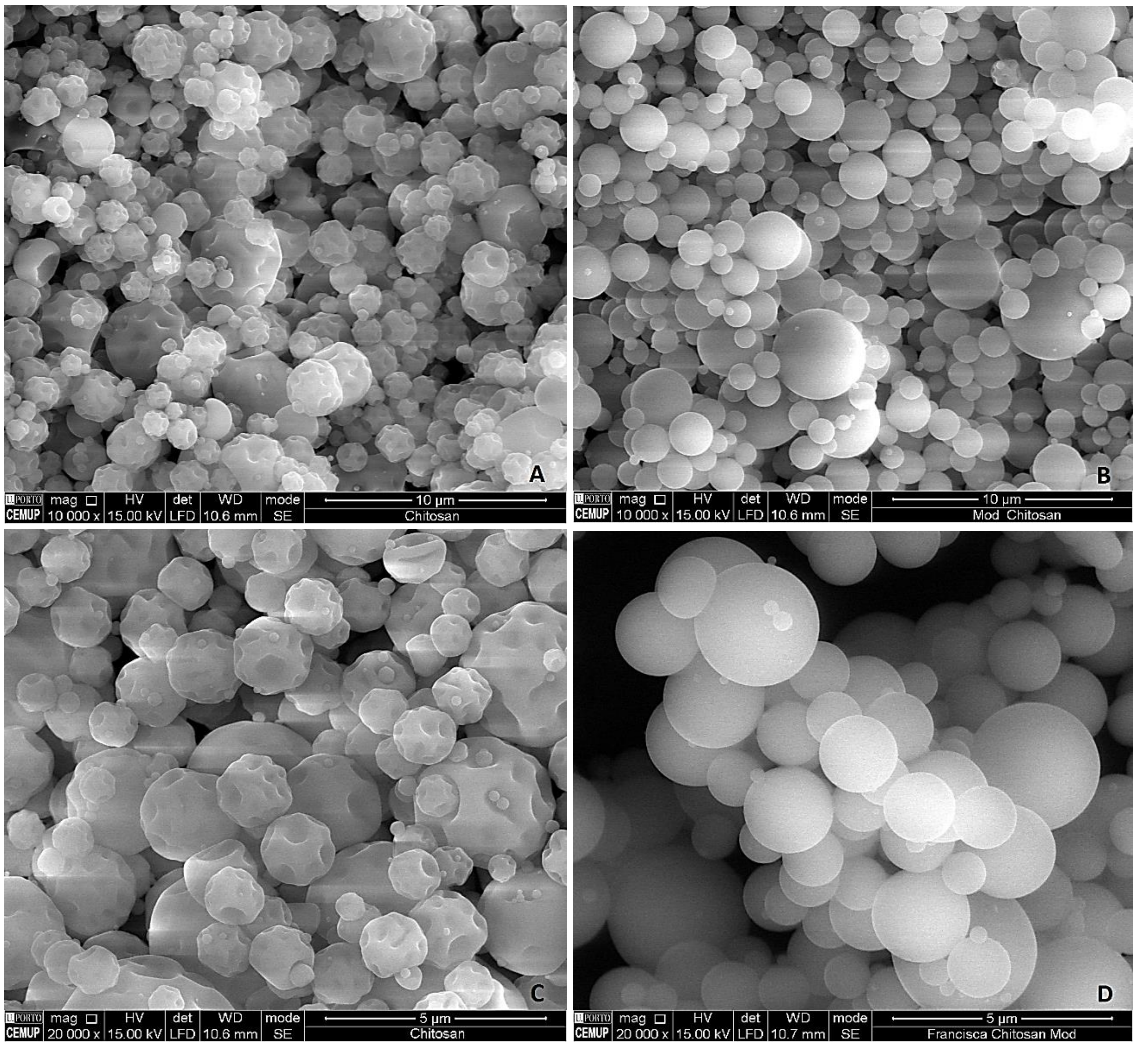


Figure 10. SEM micrographs of RA loaded chitosan (A, C) and modified chitosan (B, D) microparticles.
Amplified 10 000 (A, B) and 20 000 (C, D) times, beam intensity (HV) 15 kV, distance between the sample and the lens (WD) around 10 mm.

Particle size and size distribution are important parameters towards the development of suitable microcarriers for cosmetic purposes, as they influence active ingredient loading, the release profile and the stability of the active compound inside the microparticles. Particle size and size distribution can also influence in vivo distribution, biological fate, toxicity and the targeting ability of microparticle systems. Particle size distribution results are presented in Figure 11 and 12 and in Table 11.

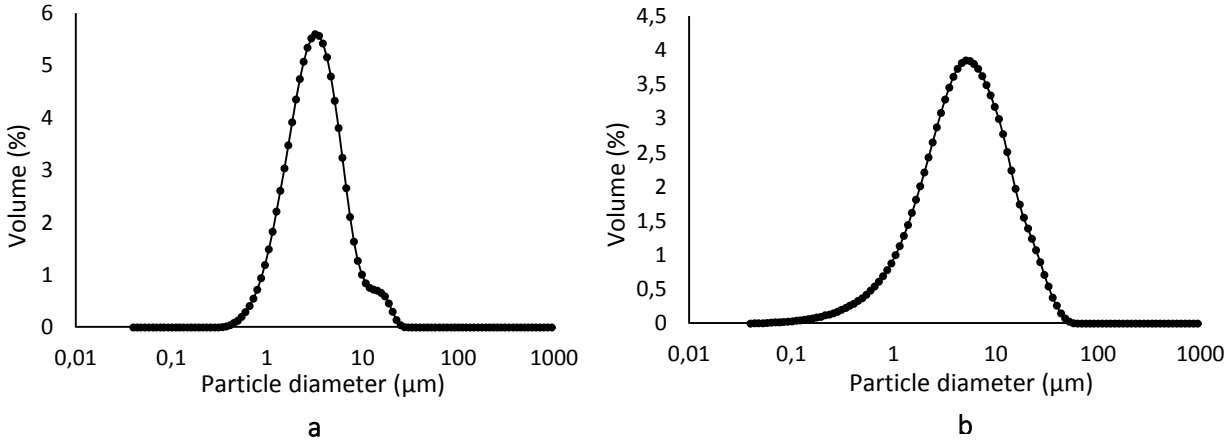


Figure 11. Size distribution in volume of RA microparticles produced with chitosan (a) and modified chitosan (b).

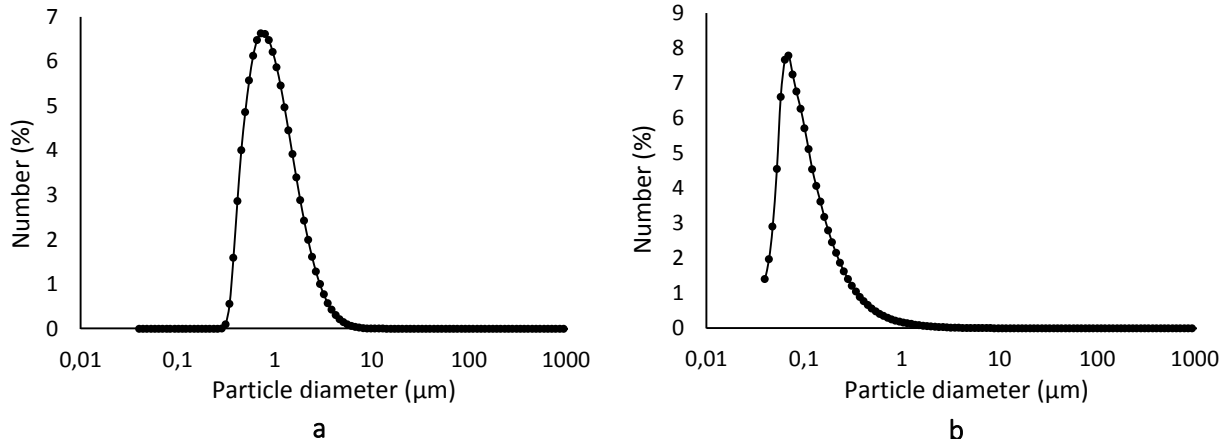


Figure 12. Size distribution in number of RA microparticles produced with chitosan (a) and modified chitosan (b).

Table 11. Particle mean diameter results by laser granulometry analysis.

Microparticles	Mean diameter (µm)	
	Differential volume	Differential number
Chitosan - RA	4.2	0.8
Modified Chitosan - RA	7.7	0.1

Similar mean diameter values were obtained for both types of microparticles produced, with distributions close to normal. The results indicated that the volume distribution of 90% of the particles is below 7.8 µm for chitosan microparticles and 17.2 µm for modified chitosan microparticles. Number distribution of 90% of the particles is below 2.0 µm for chitosan microparticles and 0.3 µm for modified chitosan microparticles. Size distribution of chitosan

particles was found to more uniform, since volume distribution is closer to number distribution and the results are close to normally distributed, except for small amounts of larger particles, which may be explained by aggregation effects. Modified chitosan microparticles exhibited a less uniform distribution, where bigger particles or agglomerates are present and a high number of nanoscale particles exist. Average particle sizes in the range of 3.20 to 59.9 and similar particle size distributions were also reported in antioxidants encapsulation by a spray drying process (Harris et al., 2011; Peres et al., 2011; Sansone et al., 2011; Kosaraju et al., 2006).

5.3. Controlled Release

The controlled release study of rosmarinic acid from the obtained particles was planned to simulate topical delivery conditions and to study the consequent chitosan and modified chitosan microparticles behaviour over a spaced period of time, until 24 h. The study of the release profile was performed in water and coconut oil in order to simulate cosmetic formulations, both balanced with the skin pH (pH ≈ 5.5). The release analysis was accomplished by evaluating the amount of rosmarinic acid released from the particles over a specified time.

The instrumental analysis for rosmarinic acid quantification was firstly performed by the HPLC method validated previously. However microparticles caused HPLC column blocking and damage, even with successive filtering and washing steps, and the controlled release results were not reliable. A chromatogram obtained after 10 min of release from chitosan and modified chitosan microparticles is presented in Figure 13, a and b, respectively. This analysis was only performed in water until 30 min of release, due to the unusable results obtained. However the chromatograms show a single peak for both types of microparticles, with retention times close to the one of rosmarinic acid. This indicates that the encapsulating agents do not interfere with the UV detection at the wavelength used, which is in accordance with chitosan UV absorption spectra reported in the literature (maximum absorption at 285 nm) (Sionkowska et al, 2013).

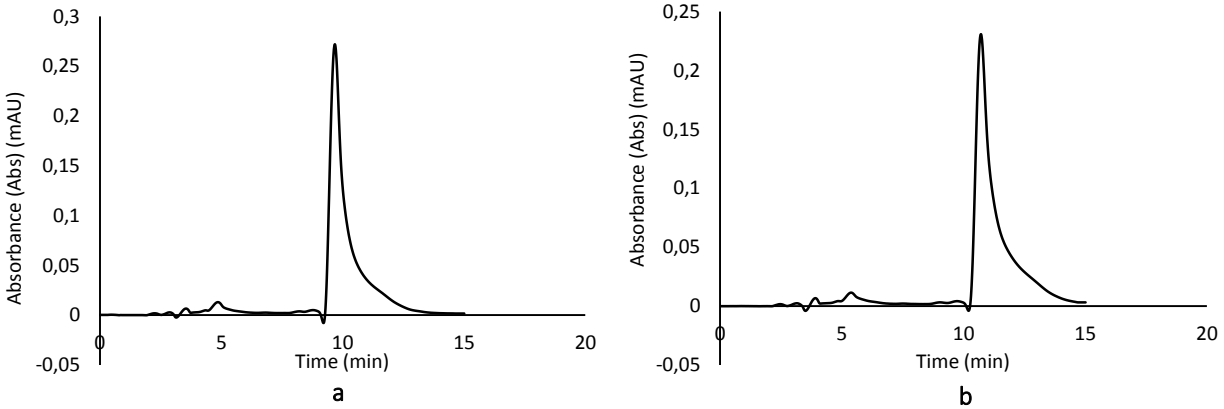


Figure 13. Chromatogram of RA-chitosan (a) and RA-modified chitosan (b) microparticles after 10 min of release in water, analysed by HPLC-DAD at 330 nm.

Considering the unusable results obtained by the chromatographic method, a UV-Vis spectrometry method was developed, validated and used for RA determination. Figure 15 compares the RA release profile from chitosan (a) and modified chitosan (b) microparticles in different mediums: water and coconut oil. Figure 16 compares the RA release profile from microparticles in water (a) and coconut oil (b) using different biopolymers: chitosan and modified chitosan. Generally the in vitro rosmarinic acid release profiles obtained for each formulation showed three phases: (1) a first initial burst release, possibly due to desorption of RA initially associated with microparticles on their surfaces by weak linkages to chitosan, (2) a constant sustained release of the remaining RA, resulting from the diffusion of RA through the polymer wall as well as its erosion, and (3) a plateau in RA concentration after complete release.

RA loaded chitosan microcapsules exhibited a fast initial release (≈ 90% after 45 min), and a sustained behaviour until complete RA release at around 4 h. This profile was observed for chitosan microparticles, both in water and coconut oil, with no significant differences (Figure 15, a). Regarding modified chitosan microparticles (Figure 15, b), a fast complete release was observed (100% at 30 min) in water, but after 4 h the profile exhibited a decrease of RA in solution. The reasons for this decrease are unknown, but there might have been adsorption of RA into the biopolymer, potentiated by interactions between RA and the modified groups of chitosan. Hydrolysis of RA is also a possibility (Figure 14), however this behaviour was not observed for chitosan microparticles in water. RA loaded modified chitosan microparticles in coconut oil showed a slower release profile, with approximately 75% of release after 2 h. This difference in behaviour in water and coconut oil is due to modified chitosan good solubility in water. In this medium, modified chitosan is well soluble, the microparticles disintegrate and RA is released. In oil, modified chitosan is less soluble and RA stays entrapped longer. The same decrease in RA in solution was observed in coconut oil for modified chitosan microparticles.

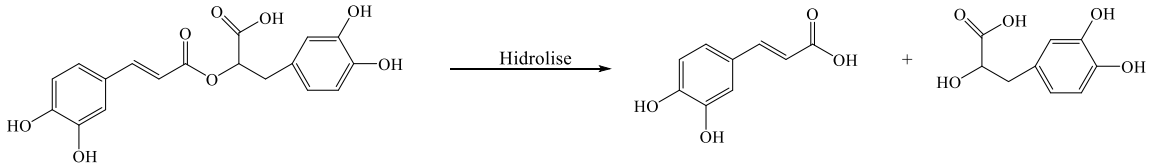


Figure 14. Hydrolysis reaction of rosmarinic acid.

Comparing the RA release profile from microparticles using the two different biopolymers, is possible to see that in water, modified chitosan exhibits a faster release (100% after 30 min) when compared to chitosan particles (95% after 2 h), due to modified chitosan better solubility in water (Figure 16, a). On the other hand, as modified chitosan is less soluble in oil, chitosan microparticles showed a faster initial release (90% at 30 min) than modified chitosan particles (75% after 2 h) in the coconut oil medium (Figure 16, b).

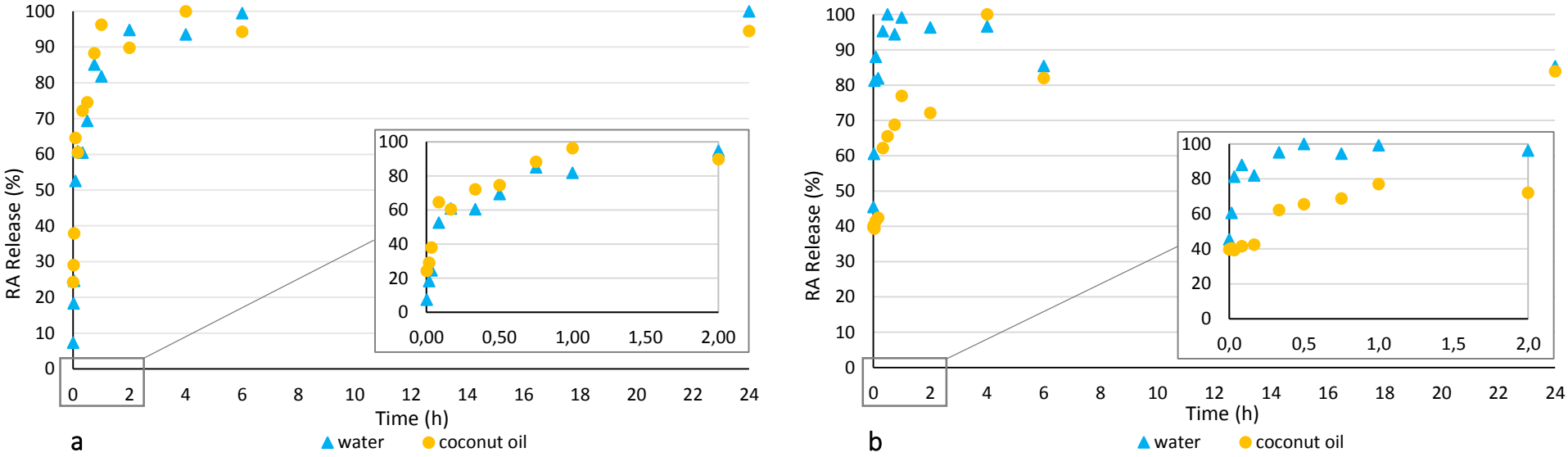


Figure 15. Comparison of the RA release profile from chitosan (a) and modified chitosan (b) microparticles in different mediums: water and coconut oil.

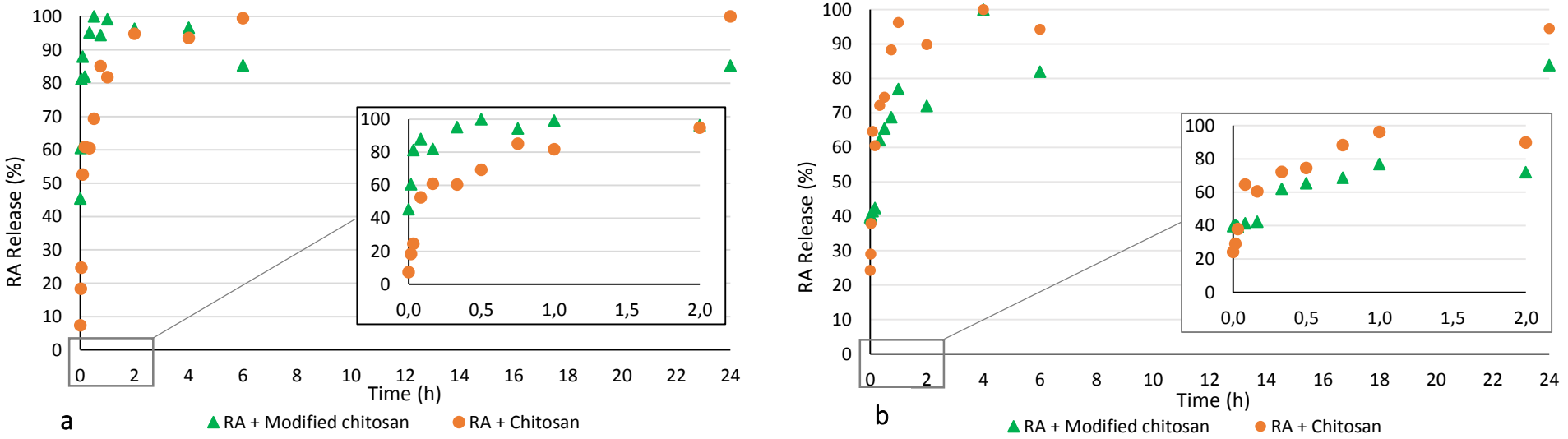


Figure 16. Comparison of the RA release profile from microparticles in water (a) and coconut oil (b) using different biopolymers: chitosan and modified chitosan.

In general a fast release profile was observed during the in vitro release assay, which was in accordance with previous data. Silva et al. (2014b) studied the release of RA and natural extracts from chitosan microparticles in PBS pH 7.4 and a fast release was also observed, with an initial burst effect in the first 30 min and reaching almost 100% of release within 60 min. Belščak-Cvitanović et al. (2011) and Visentin et al. (2012) evaluated the release of rosemary antioxidants from alginate-chitosan and poloxamers microparticles and both results showed a total release of the phenolic compounds after 60 min. However slower release profiles were observed in other studies. Deladino et al. (2008) prepared chitosan beads for the encapsulation of natural antioxidants, and the results showed a released around 35% at 3.5 h, which is significantly slower than the released observed in the present study. Dudhania et al. (2010) also demonstrated a complete release of polyphenols from chitosan particles within 4 h. Harris et al. (2011) encapsulated polyphenols in chitosan microparticles prepared by spray drying and 60% of polyphenols was delivered from microparticles after 4 h in pH 5.7 and between 40 to 45% in pH 6.5. The higher release in lower pH was due to the better solubility of chitosan. Budhiraja et al. (2014) evaluated the release of RA from niosomes in PB pH 5.5 and Bhatt et al. (2014) evaluated RA release from solid lipid nanoparticles in PBS pH 6.8. The results showed 70% and 97% of release at 12 h and 14 h, respectively. This is significantly lower than the released amount in the present study, and may be due to the differences in particle size (nano scale *versus* micro scale).

These preliminary release tests in water and coconut oil indicated that depending on the cosmetic application, aqueous or oily formulations, chitosan and modified chitosan could be used, respectively, as encapsulating agents for a more sustained release. However further studies should be done to obtain microparticles that control the release of rosmarinic acid more effectively, since for cosmetic applications a slower release is intended. The profile of compound release from microparticles can be affected by the method of encapsulation, the release medium, the pH and by the interactions between the drug, encapsulating agent and auxiliary ingredients added, and these factors should be optimized to obtain an optimum sustained release system.

5.4. Antioxidant activity

Rosmarinic acid is a phenolic compound with known antioxidant properties. The antioxidant activity of rosmarinic acid free in solution and encapsulated into chitosan and modified chitosan microparticles was tested using the ABTS radical scavenging assay, based on the reduction of a coloured oxidant (Figure 17). In this assay, by addition of sodium persulfate, ABTS is converted to its radical cation, which is blue/green in colour and absorbs light at 734 nm. The ABTS radical cation is reactive towards most antioxidants, including phenolic compounds and Trolox, a water

soluble synthetic analogue of vitamin E. During the reaction, the blue/green ABTS radical cation is reduced back to its colourless neutral form, and the reaction is monitored spectrophotometrically (Re et al. 1999; Walker et al., 2009). The reactivity of the antioxidants tested is compared to that of Trolox and expressed in µM TE.

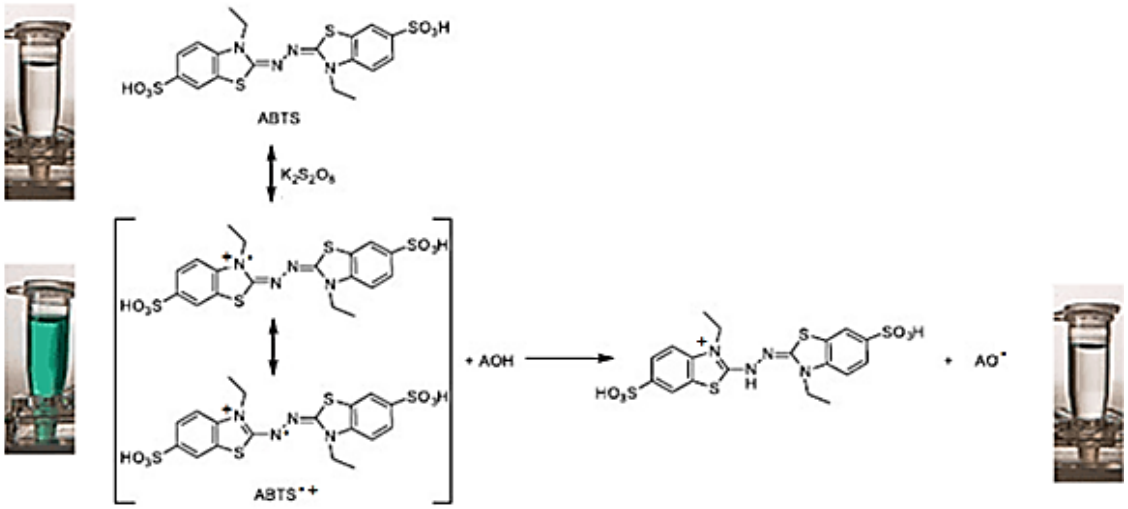


Figure 17. Oxidation of ABTS by potassium persulfate to generate radical cation $ABTS^{\bullet+}$ and its reaction with an antioxidant compound (AOH).

Antioxidant capacity results for rosmarinic acid free in solution and encapsulated into chitosan and modified chitosan microparticles are presented in Table 12:

Table 12. Antioxidant activity estimated by ABTS of RA free in solution and encapsulated into chitosan and modified chitosan microparticles.

	Antioxidant activity (µM TE) [a]
RA free in solution	342 ± 2
RA-Chitosan microparticles	216 ± 8
RA-Modified chitosan microparticles	232 ± 4

[a]Data corresponds to the mean ± standard deviation of triplicate measurements of different preparations.

Comparing the antioxidant activity of the free and encapsulated rosmarinic acid, a decrease in the antioxidant capacity can be observed after the encapsulation process, possibly due to the partial entrapment of the antioxidant. However it is well known that microcarriers protect the antioxidants from degradation, increasing their bioavailability. Nevertheless encapsulation into chitosan and modified-chitosan microparticles did not compromise the rosmarinic acid good antioxidant activity performance, showing that chitosan-based microparticles are good vehicles for RA regarding the antioxidant capacity outline. The results prove the feasibility of using chitosan particles as natural antioxidant carriers. This was in accordance with previous reports that have showed good antioxidant capacities of compounds in chitosan particles (Han et al., 2012; Amorim et al., 2010) and prepared by a spray drying method (Bakowska-Barczak et al., 2011; Peres et al., 2011).

CONCLUSIONS

In this study chitosan-based microparticles encapsulating rosmarinic acid were prepared by the spray drying method, in order to overcome its limitations in cosmetic applications. The particles were characterized, the controlled release profile was studied and the antioxidant capacity was assessed.

This was the first study to successfully report a spray-drying technique for rosmarinic acid encapsulation into chitosan and modified chitosan microparticles. Satisfactory product yields of 42.6% and 39.8%, and association efficiencies of 92.6% and 59.6% were obtained for chitosan and modified chitosan particles, respectively. The prepared microparticles showed to be spherical in shape and small sizing (4.2 and 7.7 µm) for both biopolymers tested. Chitosan particles exhibited a rough surface, while for modified chitosan some agglomeration was observed, resulting in bigger particle sizes.

An HPLC method with DAD detection for RA determination was developed and validated. All parameters were within the conditions proposed, indicating that this method was sensitive, linear (R^2 > 0.999) and precise (CV < 5%), with low detection and quantification limits (0.02 $mg.L^{-1}$ and 0.08 $mg.L^{-1}$, respectively). However this method did not prove to be suitable for RA release analysis from biopolymer microparticles, since the particles caused column blocking and damage, even after filtering and washing steps. An UV-Vis spectrometry method for RA determination was developed and validated. Validation results indicated that this method was sensitive, linear (R^2 > 0.999) and precise (CV < 5%), with low detection (0.38 $mg.L^{-1}$ in water and 0.25 $mg.L^{-1}$ in oil) and quantification (1.27 $mg.L^{-1}$ and 0.83 $mg.L^{-1}$) limits. The proposed method was used to predict the association efficiency and the release profile of RA from chitosan and modified chitosan particles.

This was the first study to investigate the controlled release profile of RA under simulated topical conditions, namely in oily matrices. Controlled release studies of RA showed a fast release (100% at 30 min) for modified chitosan particles in water, and a slower release (75% at 2 h) in oil. Chitosan particles showed a fast release (90% at 45 min) in both mediums. The results indicated that depending on the cosmetic application, aqueous or oily formulations, chitosan and modified chitosan could be used, respectively, as encapsulating agents, for a more sustained release.

Antioxidant assessment showed that, although a lower antioxidant activity was observed in the microparticles (216 ± 8 µM TE and 232 ± 4 µM TE) comparing to the free compounds (342 ± 2 µM TE), chitosan-based microparticles still maintained the rosmarinic acid good antioxidant activity performance, while allowing its protection and control of the release profile.

The efficient application of these microcarriers will certainly depend on the application. These preliminary tests reveal the potential of chitosan-based microsystems prepared by the spray-drying technique for RA delivery and prove that microparticles could be eligible vehicles to encapsulate RA and protect it, while maintaining its beneficial properties. However, if a topical delivery through cosmetic formulations is intended, a slower and more sustained release system should be optimized.

LIMITATIONS AND FUTURE WORK

Several drawbacks emerged during the development of this work, which limited the results obtained. Availability of the equipment, in specific of the HPLC, degasification system and UV-Vis spectrophotometer, was a major limitation that delayed the obtainment of results. The first phase of this work, consisting of HPLC method development, took a very long time due to low equipment availability and long running time of each sample. As mentioned, HPLC column blocking by microparticles was a major limitation that obliged to the development and validation of a new method for RA quantitative determination and delayed all the results. Rosmarinic acid instability and low solubility in water was also a limitation that forced the daily preparation of fresh intermediate and standard solutions. Regarding the antioxidant activity assessment, a DDPH radical scavenging assay was performed for comparison with the ABTS assay results, but the results were not reliable since the quality of the DDPH reagent seemed to be compromised.

As preliminary tests, the results in this study are significant and prove the success of RA microencapsulation, suggesting the need to explore new delivery systems for RA topical delivery. As discussed, if the incorporation in a topical cosmetic formulation is intended, a more controlled and sustained release system should be optimized. The profile of compound release from microparticles can be affected by the method of particles preparation, the release medium, the pH and by the interactions between the compound, encapsulating agent and auxiliary ingredients. In future work these parameters should be investigated and optimized to obtain a system with the intended properties. Different encapsulating agents could be tested, as well as other encapsulating techniques, and the addiction of cross-linking agents. Considering the low product yields obtained, possible due to the small amount of raw materials used, a scale up should be performed to minimize powder losses and to ascertain the yield of the spray drying process for the proposed encapsulation. The long-term stability of encapsulated RA over time could be investigated, by analysing RA content after a defined period of time (weeks or months). The interactions of RA with chitosan and modified chitosan during the process of encapsulation could be investigated using Fourier Transform Infrared spectroscopy analysis and thermal properties of the particles could also be evaluated. The analysis of the antimicrobial activity of encapsulated RA against selected cell lines would provide relevant information on the therapeutic potential of RA microsystems, and so would the evaluation of the anti-inflammatory capacity. It would be interesting in future work to attempt and develop a suitable extraction method for phenolic compounds, including rosmarinic acid, from rosemary (*Rosmarinus officinalis*) or other natural sources of these compounds. Encapsulation of these antioxidant extracts could be performed, and the particles could be characterized and analysed regarding release profile and antioxidant activity. Controlled release studies of microparticles encapsulating RA and rich sources thereof could be performed in emulsions mimicking cosmetic formulations or even in commercial cosmetic topical formulations for real data. Skin permeation and retention studies could be performed, using Franz diffusion cells, and in vivo studies could be done in a final stage.

REFERENCES

Abla MJ, Banga AK. (2013) Quantification of skin penetration of antioxidants of varying lipophilicity. *International Journal of Cosmetic Science*, 35: 19-26.

Alcaraz M, Alcaraz-Saura M, Achel DG, Olivares A, López-Morata JA, Castillo J. (2014) Radiosensitizing effect of rosmarinic acid in metastatic melanoma B16F10 cells. *Anticancer Research*, 34 (4): 1913-21.

Ammala A. (2013) Biodegradable polymers as encapsulation materials for cosmetics and personal care markets. *International Journal of Cosmetic Science*, 35: 113–124.

Amorim C, Couto A, Netz D, Freitas R, Bresolin T. (2010) Antioxidant Idebenone-Loaded Nanoparticles Based on Chitosan and N-Carboxymethylchitosan. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 6: 745-52.

Anwar SH, Weissbrodt J, Kunz B. (2010) Microencapsulation of Fish Oil by Spray Granulation and Fluid Bed Film Coating. *Journal of Food Science*, 75 (6): E359–E371

Aranaz I, Mengibar M, Harris R, Panos I, Miralles B, Acosta N. (2009) Functional characterization of chitin and chitosan. *Current Chemical Biology*, 3: 203-230.

Arshady, R. (1999) Microspheres, Microcapsules and liposomes: general concepts and criteria. In: *Microspheres, Microcapsules and Liposomes - Preparation and Chemical Applications*, 1: 11-45. UK: Citus Book Inc.

Aruoma OI, Spencer JPE, Rossi R, Aeschbach R, Khan A, Mahmood N, Munoz A, Murcia A, Butler J, Halliwell B. (1996) An Evaluation of the Antioxidant and Antiviral Action of Extracts of Rosemary and Provencal Herbs. *Food and Chemical Toxicology,* 34: 449—456.

Azeredo HMC. (2005) Encapsulação: aplicação à tecnologia de alimentos. *Alimentos e Nutrição*, 16 (1): 89-97.

Bakowska-Barczak A, Kolodziejczyk P. (2011) Black currant polyphenols: Their storage stability and microencapsulation. *Industrial Crops and Products*, 34 (2): 1301–1309.

Barel A, Paye M, Maibach H. (2001) *Handbook of Cosmetic Science and Technology*, New York: Marcel Dekker.

Barroso MR, Barros L, Dueñas M, Carvalho AM, Santos-Buelga C, Fernandes IP, Barreiro MF, Ferreira ICFR. (2014) Exploring the antioxidant potential of *Helichrysum stoechas* (L.) Moench phenolic compounds for cosmetic applications: chemical characterization, microencapsulation and incorporation into a moisturizer. *Industrial Crops and Products*, 53: 330–336.

Barry BW. (2002) Transdermal drug delivery. In: Aulton ME (ed), *Pharmaceutics: The Science of Dosage Form Design*, Chapter 33. London: Churchill Livingstone.

Basaga H, Tekkaya C, Acikel F. (1997) Antioxidative and Free Radical Scavenging Properties of Rosemary Extract. Lebensmittel-*Wissenschaft und –Technologie*, 30: 105-108.

Belščak-Cvitanović A, Stojanović R, Manojlović V, Komes D, Cindrić IJ, Nedović V, Bugarski B. (2011) Encapsulation of polyphenolic antioxidants from medicinal plant extracts in alginate-chitosan system enhanced with ascorbic acid by electrostatic extrusion. *Food Research International*, 44: 1094-1101.

Benita S. (2005) Microencapsulation: Methods and Industrial Applications, in *Drugs and the Pharmaceutical Sciences*, CRC Press.

Bhatt R., Singh D, Prakash A, Mishra N. (2014) Development, characterization and nasal delivery of rosmarinic acid-loaded solid lipid nanoparticles for effective management of Huntington's disease. *Drug Delivery*, early Online: 1–9.

Budhiraja A, Dhingra G. (2014) Development and characterization of a novel antiacne niosomal gel of rosmarinic acid. *Drug Delivery*, Early Online: 1-8.

Campos D, Madureira AR, Gomes AM, Sarmento B, Pintado MM. (2012) *Use of carnauba wax for the formulation of rosmarinic acid loaded solid lipid nanoparticles*. South American Symposium on Microencapsulation, April-May, Limeira, Brazil.

Campos D, Madureira AR, Gomes AM, Sarmento B, Pintado MM. (2014) Optimization of the production of solid Witepsol nanoparticles loaded with rosmarinic acid. *Colloids and Surfaces B: Biointerfaces*, 115: 109–117.

Capsutech Ltd. (2009) Cyclodextrin-containing polymers and uses thereof. Patent WO 2007072481 A2.

Carrillo JD, Tena MT. (2006) Determination of volatile compounds in antioxidant rosemary extracts by multiple headspace solid-phase microextraction and gas chromatography. *Flavour and Fragrance J*ournal, 21: 626–633.

Carvalho RNJ, Moura LS, Rosa PTV, Angela M, Meireles A. (2005) Supercritical fluid extraction from rosemary (*Rosmarinus officinalis*): Kinetic data, extract's global yield, composition, and antioxidant activity. *Journal of Supercritical Fluids*, 35: 197–204.

Cattaneo MV. (2010) Topical delivery systems based on polysaccharide microspheres. In: Rosen MR (ed), *Delivery System Handbook for Personal Care and Cosmetic Products*, 273–282. Norwich, New York: William Andrew, Inc.

Chanchal D, Swarnlata S. (2008) Novel approaches in herbal cosmetics. *Journal of Cosmetic Dermatology*, 7 (2): 89–95

Chávarri M, Marañón I, Villarán M. (2012) Encapsulation Technology to Protect Probiotic Bacteria. In: Rigobelo EC (ed), *Probiotics,* Chapter 23.

Chhotalal AK, Chavda JR, Soniwala MM. (2013) To Study Effect of Polymer, Core Ratio on Yield & Size Distribution of Microcapsules. *International Journal of Pharmamedix India*, 1 (2): 281-290.

Cocero MJ, Martin A, Mattea F, Varona S. (2009). Encapsulation and co-precipitation processes with supercritical fluids: Fundamentals and applications. *Journal of Supercritical Fluids*, 47: 546-555.

Conopco, Inc. and D/B/A Unilever. (2010) Topical composition comprising coloring antioxidants. Patent US 20090162306 A1.

Coreana Cosmetics Co. Ltd. (2001) Cosmetic material containing triple- encapsulated retinol. Patent US 6908625.

Couto RO, Conceicao EC, Chaul LT, Oliveira EMS, Martins FS, Bara MTF, Rezende KR, Alves SF, Paula JR. (2012) Spray-dried rosemary extracts: Physicochemical and antioxidant properties. *Food Chemistry*, 131: 99–105.

Del Bano MJ, Lorente J, Castillo JN, Benavente-Garcia O, Del Rio JA, Ortun A, Quirin KW, Gerard D. (2003) Phenolic Diterpenes, Flavones, and Rosmarinic Acid Distribution during the Development of Leaves, Flowers, Stems, and Roots of *Rosmarinus officinalis*. Antioxidant Activity. *Journal of Agricultural and Food Chemistry*, 51: 4247-4253.

Deladino L, Anbinder PS, Navarro AS, Martino MN. (2008) Encapsulation of natural antioxidants extracted from *Ilex paraguariensis*. *Carbohydrate Polymers*, 71 (1): 126-134.

Di Marco M, Shamsuddin S, Razak KA, Aziz AA, Devaux C, Borghi E, Levy L, Sadun C. (2010) Overview of the main methods used to combine proteins with nanosystems: absorption, bioconjugation, and encapsulation. *International Journal of Nanomedicine*, 5: 37–49.

De Vos P, Faas MM, Spasojevic M, Sikkema J. (2010) Encapsulation for preservation of functionality and targeted delivery of bioactive food components. *International Dairy Journal*, 20 (4), 292–302.

Desai KG, Liu C, Park HJ. (2006) Characteristics of vitamin C encapsulated tripolyphosphate-chitosan microspheres as affected by chitosan molecular weight. *Journal of Microencapsulation*, 23 (1): 79-90.

Desai KG, Park HJ. (2005) Preparation and characterization of drug-loaded chitosan–tripolyphosphate microspheres by spray drying. *Drug Development Research*, 64 (2):114 - 128.

Dubey R, Shami TC, Bhasker Rao K. (2009) Microencapsulation Technology and Applications. *Defence Science Journal*, 59 (1): 82-95.

Dudhania AR, Kosarajua SL. (2010) Bioadhesive Chitosan Nanoparticles: Preparation and Characterization. *Carbohydrate Polymers*, 81: 243-51.

Durand M. (1995) Method for the protection of dihydroxyacetone, a dihydroxyacetone protected by this method, and a cosmetic product containing such a protected dihydroxyacetone. Patent US 5458872 A.

El Maghraby GM, Barryc BW, Williams AC. (2008) Liposomes and skin: From drug delivery to model membranes. *European Journal of Pharmaceutical Sciences*, 34 (4-5): 203–222.

Elias PM. (2004) The epidermal permeability barrier: from the early days at harvard to emerging concepts. *Journal of Investigative Dermatology*, 122 (2): 36-39.

Estevinho BN, Damas AM, Martins P, Rocha F. (2012) Study of the Inhibition Effect on the Microencapsulated Enzyme β-galactosidase. *Environmental Engineering and Management Journal*, 11: 1923–1930.

Estevinho BN, Damas AM, Martins P, Rocha F. (2014a) The Influence of Microencapsulation with a Modified Chitosan (Water Soluble) on β-galactosidase Activity. *Drying Technology Journal*, 32: 1575–1586.

Estevinho BN, Damas AM, Martins P, Rocha F. (2014b) Microencapsulation of β-galactosidase with different biopolymers by a spray-drying process. *Food Research International Journal*, 64: 134–140.

Estevinho BN, Rocha F, Santos L, Alves A. (2013a) Microencapsulation with chitosan by spray drying for industry applications – A review. *Trends in Food Science & Technology Journal*, 31: 138–155.

Estevinho BN, Rocha F, Santos L, Alves A. (2013b) Using Water Soluble Chitosan for Flavour Microencapsulation in Food Industry. *Journal of Microencapsulation*, 30: 571–579.

Erkan N, Ayranci G, Ayranci E. (2008) Antioxidant activities of rosemary (*Rosmarinus officinalis L.*) extract, blackseed (*Nigella sativa* L.) essential oil, carnosic acid, rosmarinic acid and sesamol. *Food Chemistry,* 110: 76–82.

Euromonitor. (2011) Beauty and personal care 2011: corporate strategies in and beyond the BRICs. In: *Euromonitor International* (Ed).

Fadel O, Ghazi Z, Mouni L, Benchat N, Ramdani M, Amhamdi H, Wathelet JP, Asehraou A, Charof R. (2011) Comparison of Microwave-Assisted Hydrodistillation and Traditional Hydrodistillation Methods for the *Rosmarinus eriocalyx* essential oils from Eastern Morocco. *Journal of Materials and Environmental Science,* 2 (2): 112-117.

Fairhurst D, Loxley A. (2008) Micro- and Nano-encapsulation of Water- and Oil-soluble Actives for Cosmetic and Pharmaceutical Applications. In: *Cosmetic Delivery Systems*, Chapter 1. Bethlehem, PA: Particle Sciences Inc.

Fecka I, Raj D, Krauze-Baranowska M. (2007) Quantitative Determination of Four Water-Soluble Compounds in Herbal Drug from Lamiaceae Using Different Chromatographic Techniques. *Chromatographia*, 66: 87–93.

Fernandes RVB, Borges SV, Botrel DA. (2014). Gum arabic/starch/maltodextrin/inulin as wall materials on the microencapsulation of rosemary essential oil. *Carbohydrate Polymers,* 101: 524– 532.

Fernandes RVB, Borges SV, Botrel DA. (2013a) Influence of spray drying operating conditions on microencapsulated rosemary essential oil properties. *Ciência e Tecnologia de Alimentos*, 33 (1): 171-178.

Fernandes RVB, Borges SV, Botrel DA, Silva EK, Costa JMG, Queiroz F. (2013b) Microencapsulation of Rosemary Essential Oil: Characterization of Particles. *Drying Technology*, 31: 1245–1254.

Fernandez-Saiz P, Lagaron JM, Ocio MJ. (2009) Optimization of the Film-Forming and Storage Conditions of Chitosan as an Antimicrobial Agent. *Journal of Agriculture and Food Chemistry*, 57: 3298–307.

Fidalgo A, Ciriminna R, Lopes L, Pandarus V, Béland F, Ilharco LM, Pagliaro M. (2013) The sol-gel entrapment of noble metals in hybrid silicas: A molecular insight. *Chemistry Central Journal*, 7: 161-169.

Flynn GL. (2002) Cutaneous and transdermal delivery-processes and systems of delivery. In: Banker GS, Rhodes CT (ed), *Modern Pharmaceutics*, Chapter 8. New York: Marcel Dekker.

Forster M, Bolzinger MA, Fessi H, Briançon S. (2009) Topical delivery of cosmetics and drugs. Molecular aspects of percutaneous absorption and delivery. *European Journal of Dermatology*, 19: 309–323.

Freitas S, Walz A, Merkle HP, Gander B. (2003) Solvent extraction employing a static micromixer: a simple, robust and versatile technology for the microencapsulation of proteins. *Journal of Microencapsulation*, 20: 67-85.

Fu X, Ping Q, Gao Y. (2005) Effects of formulation factors on encapsulation efficiency and release behaviour in vitro of huperzine A-PLGA microspheres. *Journal of Microencapsulation*, 22 (1): 57 – 66.

Gachkar L, Yadegari D, Rezaei MB, Taghizadeh M, Alipoor AS, Rasooli I. (2007). Chemical and biological characteristics of *Cuminum cyminum* and *Rosmarinus officinalis* essential oils. *Food Chemistry*, 102: 898–904.

Gallarate M, Carlotti ME, Trotta M, Bovo S. (1999) On the stability of ascorbic acid in emulsified systems for topical and cosmetic use. *International Journal of Pharmaceutics*, 188 (2): 233-241.

Gander B, Merkle HP, Nguyen VP, Ho NT. (1995) A new thermodynamic model to predict protein encapsulation efficiency in poly(lactide) microspheres. *Journal of Physical Chemistry*, 99 (43): 16144–16148.

Garcıa MA, Pinotti A, Martino M, Zaritzky N. (2009) Electrically Treated Composite Films Based on Chitosan and Methylcellulose Blends. *Food Hydrocolloids*, 23: 722-8.

Gharsallaoui A, Roudaut G, Chambin O, Voilley A, Saurel R. (2007) Applications of spray-drying in microencapsulation of food ingredients: an overview. *Food Research International*, 40: 1107–1121.

Ghosh SK. (2006) Functional Coatings and Microencapsulation: A General Perspective in Functional Coatings. In: Ghosh SK (ed), *Polymer Microencapsulation*. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA.

Gudzenko A. (2013) Development and validation of a method for the determination of rosmarinic acid in *Mentha piperita* L. using solid-phase extraction and RP-HPLC with photodiode array detection. *Journal of Chemical and Pharmaceutical Research*, 5 (9): 40-45.

Gutcho M. (1976) *Microcapsules and microencapsulation techniques (Chemical technology Review)*. USA: Noyes Data Corporation.

Haddadi A, Aboofazeli R, Erfan M, Farboud ES. (2008) Topical delivery of urea encapsulated in biodegradable PLGA microparticles: O/W and W/O creams. *Journal of Microencapsulation*, 25 (6): 379–386.

Hammad U, Hemlata N, Asif M, Nainar M. (2011) Microencapsulation: Process, Techniques and Applications. *International Journal of Research in Pharmaceutical and Biomedical Sciences*, 2 (2): 474-481.

Han L, Du L-B, Kumar A, Jia H-Y, Liang X-J, Tian Q. (2012) Inhibitory Effects of Trolox-Encapsulated Chitosan Nanoparticles on Tertbutylhydroperoxide Induced RAW264.7 Apoptosis. *Biomaterials*, 33:8517-28.

Harding CR. (2004) The *stratum corneum*: structure and function in health and disease. *Dermatology and Therapy,* 17: 6–15.

Harris H, Lecumberri E, Mateos-Aparicio I, Mengíbar M, Heras A. (2011) Chitosan nanoparticles and microspheres for the encapsulation of natural antioxidants extracted from *Ilex paraguariensis. Carbohydrate Polymers*, 84 (2): 803–806.

He W, Guo X, Xiao L, Feng M. (2009) Study on the mechanisms of chitosan and its derivatives used as transdermal penetration enhancers. *International Journal of Pharmaceutics*, 382: 234–243.

Hernández-Hernández E, Ponce-Alquicira E, Jaramillo-Flores ME, Legarreta IG. (2009) Antioxidant effect rosemary (*Rosmarinus officinalis* L.) and oregano (*Origanum vulgare* L.) extracts on TBARS and colour of model raw pork batters. *Meat Science*, 81: 410-417.

Hirech K, Payan S, Carnelle G, Brujes L, Legrand J. (2003). Microencapsulation of an insecticide by interfacial polymerisation. *Powder Technology*, 130: 324-330.

Hooker CW, Lott WB, Harrich D. (2011) Inhibitors of human immunodeficiency virus type 1 reverse transcriptase target distinct phases of early reverse transcription. *Journal of virology,* 75 (7): 3095-3104.

Hossan MS, Rahman S, Bashar ABMA, Jahan R, Al-Nahain A, Rahmatullah M. (2014) Rosmarinic acid: A Review of its Anticancer Action. *World Journal of Pharmacy and Pharmaceutical Sciences*, 3 (9): 57-70.

Ionita P, Dinoiu V, Munteanu C, Turcu IM, Tecuceanu V, Zaharescu T, Oprea E, Ilie C, Anghel D. (2014) Antioxidant activity of rosemary extracts in solution and embedded in polymeric systems. *Chemical Papers*, 69 (6): 872–880.

Jain S, Tiwari A.K, Jain NK. (2006) Topical products. In: Jain NK (ed), *Pharmaceutical Product Development*, Chapter 7. New Delhi: CBS Publishers.

Jafari SM, Assadpoor E, He Y, Bhandari B. (2008) Encapsulation efficiency of food flavours and oils during spray drying. *Drying Technology*, 26 (7): 816-835.

Jafari SM, He Y, Bhandari B. (2007) Encapsulation of nanoparticles of d-Limonene by spray drying: role of emulsifiers and emulsifying techniques. *Drying Technology*, 25 (6): 1079-1089.

Jyothi N, Prasanna M, Prabha S, Ramaiah P, Srawan G, Sakarkar S. (2010) Microencapsulation Techniques, Factors Influencing Encapsulation Efficiency. *Journal of Microencapsulation*, 27 (3): 187–197.

Kaur I, Kapila M, Agrawal R. (2007) Role of novel delivery systems in developing topical antioxidants as therapeutics to combat photoageing. *Ageing Research Reviews,* 6 (4): 271–288.

Kaur L, Sharma S, Guleri T. (2013) Microencapsulation: A New Era in Noval Drug Delivery. *International Journal of Pharmaceutical and Bio-science*, 2 (2): 456-468.

Kelsey NA, Wilkins HM, Linseman DA. (2010) Nutraceutical antioxidants as novel neuroprotective agents. *Molecules,* 15:7792–814.

Kim HJ, Kim TH, Kang KC, Pyo HB, Jeong HH. (2010) Microencapsulation of rosmarinic acid using polycaprolactone and various surfactants*. International Journal of Cosmetic Science*, 32: 185–191.

Kim NS, Lee DS. (2001) Characterization of Rosemary Fragrances by Solid Phase Micro-extraction and GC-MS. *Analytical Sciences*, 17: 383-386.

Kosaraju S, D'ath L, Lawrence A. (2006) Preparation and characterisation of chitosan microspheres for antioxidant delivery. *Carbohydrate Polymers*, 64 (2): 163–167.

L'Oreal. (1998) Composition comprising an aqueous dispersion of lipid vesicles encapsulating a UV screening agent with acidic functionality and uses in topical application. Patent US 5759526 A.

Lam PL, Gambari R. (2014) Advanced progress of microencapsulation technologies: In vivo and in vitro models for studying oral and transdermal drug deliveries. *Journal of Controlled Release,* 178: 25–45.

Lee KY, Mooney DJ. (2012) Alginate: properties and biomedical applications. *Progress in Polymer Science,* 37 (1): 106–126.

Liu T, Sui X, Zhang R, Yang L, Zu Y, Zhang L, Zhang Y, Zhang Z. (2011) Application of ionic liquids based microwave-assisted simultaneous extraction of carnosic acid, rosmarinic acid and essential oil from *Rosmarinus officinalis. Journal of Chromatography A*, 1218: 8480– 8489.

Luis JC, Pérez RM, González FV. (2007) UV-B radiation effects on foliar concentrations of rosmarinic and carnosic acids in rosemary plants*. Food Chemistry*, 101: 1211-1215.

Lumsdon SO, Friedmann TE, Green JH. (2005) Encapsulation of oils by coacervation. WIPO Patent WO/2005/105290.

Madureira AR, Campos D, Fonte P, Nunes S, Reis F, Gomes AM, Sarmento B, Pintado MM. (2015) Characterization of solid lipid nanoparticles produced with carnauba wax for rosmarinic acid oral delivery. *The Royal Society of Chemistry*, 5: 22665–22673.

Martins I, Barreiro M, Coelho M, Rodrigues A. (2014) Microencapsulation of essential oils with biodegradable polymeric carriers for cosmetic applications. *Chemical Engineering Journal*, 245: 191–200.

Martins I, Rodrigues S, Barreiro F, Rodrigues A. (2010) Polylactide-based thyme oil microcapsules production: evaluation of surfactants*. Industrial and Engineering Chemistry Research*, 50 (2): 898–904.

Martins M, Rodrigues A, Barreiro F, Rodrigues S. (2009) Microencapsulation of thyme oil by coacervation. *Journal of Microencapsulation*, 26 (8):667-75.

Matsuda H, Arima H. (1999) Cyclodextrins in transdermal and rectal delivery. *Advanced Drug Delivery Reviews,* 36 (1): 81–99.

Maybelline Intermediate Company. (1999) Skin revitalizing makeup composition. Patent EP 0796077 A1.

Michael H. (2009) Chemical product engineering - The 3rd paradigm. *Computers & Chemical Engineering*, 33 (5): 947–953.

Mishra N, Goyal AK, Khatri K. (2008) Biodegradable polymer based particulate carrier(s) for the delivery of proteins and peptides. *Anti-Inflammatory & Anti-Allergy Agents in Medicinal Chemistry,* 7: 240–251.

Moghimi HR, Williams AC, Barry BW. (1999) *Stratum corneum* and barrier performance; a model lamellar structural approach. In: Bronaugh RL, Maibach HI (eds), *Percutaneous Absorption*, 515–553. New York: Marcel Dekker.

Müller R, Mader K, Gohla S. (2000) Solid lipid nanoparticles (SLN) for controlled drug delivery – a review of the state of the art. *European Journal of Pharmaceutics and Biopharmaceutics*, 50 (1): 161–177

Müller R, Radtke M, Wissing S. (2002) Nanostructured Lipid Matrices for Improved Microencapsulation of Drugs. *International Journal of Pharmaceutics*, 242: 121-128.

Muzzarelli, RA. (1998) Management of hypercholesterolemia and overweight by oral administration of chitosan. *Biomedical Health Research*, 16, 135–142.

Nack H. (1970) Microencapsulation Techniques Applications and Problems. *Journal of the Society of Cosmetic Chemists*, 21: 85-98.

Nguyen-Ngoc H, Tran-Minh C. (2007) Sol-gel process for vegetal cell encapsulation. *Materials Science and Engineering C*, 27: 607-611.

Nikkola J, Virtanen S, Pelto J, Munter T, Ropponen J, Mangs J, Mahlberg R. (2014) *Novel responsive surfaces based on active hybrid coatings utilizing encapsulation technologies*. Research Report VTT-R-04314-14.

Okoh OO, Sadimenko AP, Afolayan AJ. (2010) Comparative evaluation of the antibacterial activities of the essential oils of *Rosmarinus officinalis* L. obtained by hydrodistillation and solvent free microwave extraction methods. *Food Chemistry,* 120: 308-312.

Oresajo C, Pillai S, Manco M, Yatskayer M, McDaniel D. (2012) Antioxidants and the skin: Understanding formulation and efficacy. *Dermatologic Therapy*, 25: 252-259.

Osakabe N, Yasuda A, Natsume M, Yoshikawa T. (2004) Rosmarinic acid inhibits epidermal inflammatory responses: anticarcinogenic effect of *Perilla frutescens* extract in the murine two-stage skin model. *Carcinogenesis*, 25 (4): 549-557.

Pardeike J, Hommoss A, Müller R. (2009) Lipid nanoparticles (SLN, NLC) in cosmetic and pharmaceutical dermal products. *International Journal of Pharmaceutics,* 366 (1-2): 170-84.

Pawar KR, Babu RJ. (2010) Polymeric and lipid-based materials for topical nanoparticle delivery systems. *Critical Reviews in Therapeutic Drug Carrier Systems*, 27 (5): 419–459

Pedro AS, Cabral-Albuquerque E, Ferreira D, Sarmento B. (2009) Chitosan: an option for development of essential oil delivery systems for oral cavity care. *Carbohydrate Polymers*, 76 (4) 501–508.

Peres I, Rocha S, Gomes J, Morais S, Pereira C, Coelho M. (2011) Preservation of Catechin Antioxidant Properties Loaded in Carbohydrate Nanoparticles. *Carbohydrate Polymers*, 86: 147-53.

Petersen M. (2013) Rosmarinic acid: new aspects. *Phytochemistry Reviews*, 12: 207–227.

Petersen M, Abdullah Y, Benner J, Eberle D, Gehlen K, Hücherig S, Janiak V, Kim KH, Sander M, Weitzel C, Wolters S. (2009) Evolution of rosmarinic acid biosynthesis. *Phytochemistry*, 70 (15-16): 1663-79.

Petersen M, Häusler E, Karwatzki B, Meinhard J. (1993) Proposed biosynthetic pathway for rosmarinic acid in cell cultures of *Coleus blumei*. *Planta*, 189 (1): 10-14.

Poljsak B, Dahmane R, Godic A. (2013) Skin and antioxidants. *Journal of Cosmetic and Laser Therapy,* 15: 107-113.

Psotova J, Svobodova A, Kolarova H, Walterova D. (2006) Photoprotective properties of *Prunella vulgaris* and rosmarinic acid on human keratinocytes. *Journal of Photochemistry and Photobiology B: Biology*, 84, 167-174.

R.P. Scherer Corporation. (1996) Topical application emulsions. Patent US 5587149 A.

Ramu S., Srinivasa D., Surekha N., Ramakrishna G. (2014) Formulation and Evaluation of Microencapsulated Suspension of Ofloxacin. *International Journal of Pharma Research and Health Sciences*, 2 (4): 307-315.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, 26 (9-10): 1231-7.

Rein H. (1924) Experimental electroendosmotic studies on living human skin. *Z Biol Journal*, 81: 125-140

Robert P, Gorena T, Romero N, Sepulveda E, Chavez J, Saenz C. (2010) Encapsulation of polyphenols and anthocyanins from pomegranate (*Punica granatum*) by spray drying. *International Journal of Food Science and Technolog*y, 45: 1386-1394.

Rosen M. (2005) *Delivery System Handbook for Personal Care and Cosmetic Products: Technology, Applications and Formulations, Personal Care and Cosmetic Technology*. Norwich, New York: William Andrew, Inc.

Sanbongi C, Takano H, Osakabe N, Sasa N, Natsume M, Yanagisawa R, Inoue K, Sadakane K, Ichinose T, Yoshikawa T. Rosmarinic acid in perilla extract inhibits allergic inflammation induced by mite allergen, in mouse model. *Clin. Exp. Allergy*, 34: 971–977

Sánchez-Campillo M, Gabaldon JA, Castillo J, Benavente-García O, Del Baño MJ, Alcaraz M, Vicente V, Alvarez N, Lozano JA. (2009) Rosmarinic acid, a photo-protective agent against UV and other ionizing radiations. *Food and Chemical Toxicology*, 47: 386-392.

Sansone F, Mencherini T, Picerno P, d'Amore M, Aquino R, Lauro MR. (2011) Maltodextrin/pectin microparticles by spray drying as carrier for nutraceutical extracts. *Journal of Food Engineering*, 105 (3): 468–476.

Sashiwa H, Aiba S. (2004) Chemically Modified Chitin and Chitosan as Biomaterials. *Progress in Polymer Science,* 29: 887-908.

Sashiwa H, Kawasaki N, Nakayama A. (2002) Chemical modification of chitosan. Synthesis of water-soluble chitosan derivatives by simple acetylation. *Biomacromolecules*, 3 (5): 1126-1128.

Selvaraj S, Karthikeyan J, Saravanakumar N. (2012) Chitosan loaded microspheres as an ocular delivery system for acyclovir. *International Journal of Pharmacy & Pharmaceutical Sciences*, 4 (1): 125-132.

Senuma Y, Lowe C, Zweifel Y, Hilborn G, Marison I. (2000) Alginate Hydrogel Microspheres and Microcapsules Prepared by Spinning Disk Atomization. *Biotechnology and Bioengineering*, 67 (5): 616-622.

Shaklee Corporation. (2001) Improved stable topical ascorbic acidcompositions. Patent EP 1096922 A1.

Shinde T, Sun-Waterhouse D, Brooks J. (2014) Co-extrusion Encapsulation of Probiotic *Lactobacillus acidophilus* Alone or Together with Apple Skin Polyphenols: An Aqueous and Value-Added Delivery System Using Alginate. *Food and Bioprocess Technology*, 7 (6): 1581-1596.

Silva P, Fries L, Menezes C, Holkem A, Schwan C, Wigmann E, Bastos J, Silva C. (2014a) Microencapsulation: concepts, mechanisms, methods and some applications in food technology. *Ciência Rural*, 44 (7): 1304-1311.

Silva SB. (2014) *Chitosan-based nanomedicine for rosmarinic acid ocular delivery*. Thesis presented to obtain the PhD degree in Pharmaceutical Sciences, Pharmaceutical Technology Specialty, Faculty of Pharmacy of University of Porto.

Silva SB, Amorim M, Fonte P, Madureira R, Ferreira D, Pintado M, Sarmento B. (2014b) Natural extracts into chitosan nanocarriers for rosmarinic acid drug delivery. *Pharmaceutical Biology*, 53 (5): 642-52.

Silva SB, Fernandes J, Tavira F. (2011) The potential of chitosan in drug delivery systems. In: Ferguson AN, O'Neill AG (eds), *Focus on Chitosan Research*. New York: Nova Publishers.

Sinko PJ. (2006) Chemical Kinetics and Stability. In: *Martin's Physical Pharmacy and Pharmaceutical Sciences*, Chapter 14. Baltimore: Lippincott Williams & Wilkins.

Sionkowska A, Płanecka A, Lewandowska K, Kaczmarek B, Szarszewska P. (2013) influence of UV-irradiation on molecular weight of chitosan. *Progress on Chemistry and Application of Chitin and its Derivatives*, 18: 21–28.

Soest JGV. (2007) Encapsulation of fragrances and flavours: a way to control odour and aroma in consumer products. In: Berger RG (ed), *Flavours and Fragrances – Chemistry, Bioprocessing and Sustainability*. Germany: Springer.

Stansbury J, Saunders P, Winston D, Zampieron ER. (2012) Rosmarinic Acid as a Novel Agent in the Treatment of Autoimmune Disease. *Journal of Restorative Medicine*, 1: 112-116.

Stevanovic M, Savic J, Jordovic B, Uskokovic D. (2007) Fabrication, *in vitro* degradation and the release behaviours of poly(dllactide-co-glycolide) nanospheres containing ascorbic acid. *Colloids and Surfaces*, 59 (2): 215–223.

Sui X, Liu T, Ma C, Yang L, Zu Y, Zhang L, Wang H. (2012) Microwave irradiation to pretreat rosemary (*Rosmarinus* officinalis L.) for maintaining antioxidant content during storage and to extract essential oil simultaneously. *Food Chemistry*, 131: 1399-1405.

Sundaram S, Tripathi A, Gupta DK. (2010) Metabolic modeling of Rosmarinic acid biosynthetic pathway. *Bioinformation,* 5 (4): 168-172.

Sunsmart, Inc. and Sibmicro Encapsulation Technologies, Inc. (1998) Composite UV sunblock compositions. Patent US 5733531 A.

Suraweera RK, Pasansi HGP, Herath HMDR, Wickramaratne DBM, Sudeshika SHT, Niyangoda D, Sakeena MHF. (2014) Formulation and Stability Evaluation of Ketoprofen Loaded Virgin Coconut Oil based Creamy Emulsion. *International Journal of Pharmacy and Pharmaceutical Sciences*, 6 (8): 249-254.

Swarup V,Ghosh J, Ghosh S, Saxena A, Basu A. (2007) Antiviral and anti-inflammatory effects of rosmarinic acid in an experimental murine model of Japanese encephalitis. *Antimicrobial agents and chemotherapy,* 51 (9): 3367-3370.

Tarimci N. (2011) Cyclodextrins in the cosmetic field. In: Bilensoy E. (ed), *Cyclodextrins in Pharmaceutics, Cosmetics, and Biomedicine: Current and Future Industrial Applications*, 131–144. Hoboken, New Jersey: John Wiley & Sons, Inc.

Teodoro RAR, Fernandes RVB, Botrel DA, Borges SV, Souza AU. (2014) Characterization of Microencapsulated Rosemary Essential Oil and Its Antimicrobial Effect on Fresh Dough. *Food Bioprocess Technology,* 7: 2560–2569.

Tfouni SV, Carreiro LB, Teles CR, Furlani RPZ, Cipolli KMVB, Camargo MCR. (2014) Caffeine and chlorogenic acids intake from coffee brew: influence of roasting degree and brewing procedure. *International Journal of Food Science & Technology*, 49 (3): 747–752.

Thaipong K, Boonprakoba U, Crosbyb K, Cisneros-Zevallosc L, Byrne DH. (2006) Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *Journal of Food Composition and Analysis*, 19: 669–675.

The Herb Society of America. (2009) Rosemary fact sheet. Available at: http://www.herbsociety.org/ [Accessed in April, 2015]

Tokoro A, Tatewaki N, Suzuki K, Mikami T, Suzuki S, Suzuki M. (1988) Growth-inhibitory effect of hexa-N-acetylchitohexaose and chitohexaose against Meth-A solid tumor. *Chemical & Pharmaceutical Bulletin*, 36: 784–790

Türkoğlu A, Burub ME, Mercan N. (2007) Antioxidant and Antimicrobial Activity of Russula delica Fr: An Edidle Wild Mushroom. *Eurasian Journal of Analytical Chemistry*, 2 (1): 54-67.

Visentin A, Rodríguez-Rojo S, Navarrete A, Maestri D, Cocero MJ. (2012) Precipitation and encapsulation of rosemary antioxidants by supercritical antisolvent process. *Journal of Food Engineering*, 109: 9–15.

Walker RB, Everette JD. (2009) Comparative reaction rates of various antioxidants with ABTS radical cation. *Journal of Agricultural and Food Chemistry*, 57 (4): 1156-61.

Wang H, Provan GJ, Helliwell K. (2004) Determination of rosmarinic acid and caffeic acid in aromatic herbs by HPLC. *Food Chemistry*, 87: 307-311.

Wang W, Wu N, Zu YG, Fu YJ. (2008) Antioxidative activity of *Rosmarinus officinalis* L. essential oil compared to its main componentes. *Food Chemistry*, 108: 1019-1022.

Wesselingh JA, Kill S, Vild ME. (2007) *Design & Development of Biological, Chemical, Food and Pharmaceutical Products*. Chichester: Wiley Ed.

Wiechers J. (2005) Optimizing skin delivery of active ingredients from emulsions: from theory to practice. In: Rosen MR (ed), *Delivery System Handbook for Personal Care and Cosmetic Products-Technology, Applications, and Formulations*, Chapter 20. Norwich, New York: William Andrew, Inc.

Wiechers J. (2008) *Science and Applications of Skin Delivery Systems.* USA: Allured Publishing Corporation.

Wille JJ. (2006) Thixogel: a starch matrix encapsulation technology for topical drug and cosmetic delivery. In: Wille JJ (ed), *Skin Delivery Systems: Transdermals, Dermatologicals and Cosmetic Actives*, 223–245. Oxford: Blackwell Publishing.

Wilson N, Shah NP. (2007) Review Paper: Microencapsulation of Vitamins. *ASEAN Food Journal*, 14 (1): 1-14.

Zheng W, Wang SY. (2001) Antioxidant Activity and Phenolic Compounds in Selected Herbs. *Journal of Agricultural and Food Chemistry*, 49: 5165-5170.

Zibetti AW, Aydi A, Livia MA, Bolzan A, Barth D. (2013) Solvent extraction and purification of rosmarinic acid from supercritical fluid extraction fractionation waste: Economic evaluation and scale-up. *The Journal of Supercritical Fluids*, 83: 133-145.

Zu G, Zhang R, Yang L, Ma C, Zu Y, Wang W, Zhao C. (2012) Ultrasound-Assisted Extraction of Carnosic Acid and Rosmarinic Acid Using Ionic Liquid Solution from *Rosmarinus officinalis. International Journal of Molecular Sciences*, 13: 11027-11043.

APPENDIX

A. Biosynthetic pathway of rosmarinic acid

Phenylalanine (**1**) is transformed to 4-coumaroyl-CoA (**2**) by the enzymes of the general phenylpropanoid pathway: phenylalanine ammonia-lyase (EC 4.3.1.5) (**a**), cinnamic acid 4-hydroxylase (EC 1.14.13.11) (**b**) and hydroxycinnamic acid:CoA ligase (EC 6.2.1.12) (**c**). Tyrosine (**3**) is metabolized to 4-hydroxyphenyllactate (**4**) by tyrosine aminotransferase (EC 2.6.1.5) (**d**) and hydroxyphenylpyruvate reductase (**e**). The ester (**5**) can be formed from 4-coumaroyl-CoA and 4-hydroxyphenyllactate by the catalytic activity of rosmarinic acid synthase (**f**) with concomitant release of CoA. Microsomal hydroxylase activities introduce the hydroxyl groups at positions 3 and 3′ of the aromatic rings of the ester 4-coumaroyl-4′-hydroxyphenyllactate giving rise to rosmarinic acid (**6**) (Figure A1) (Petersen, 2013; Sundaram, 2010; Petersen et al., 2009, 1993).

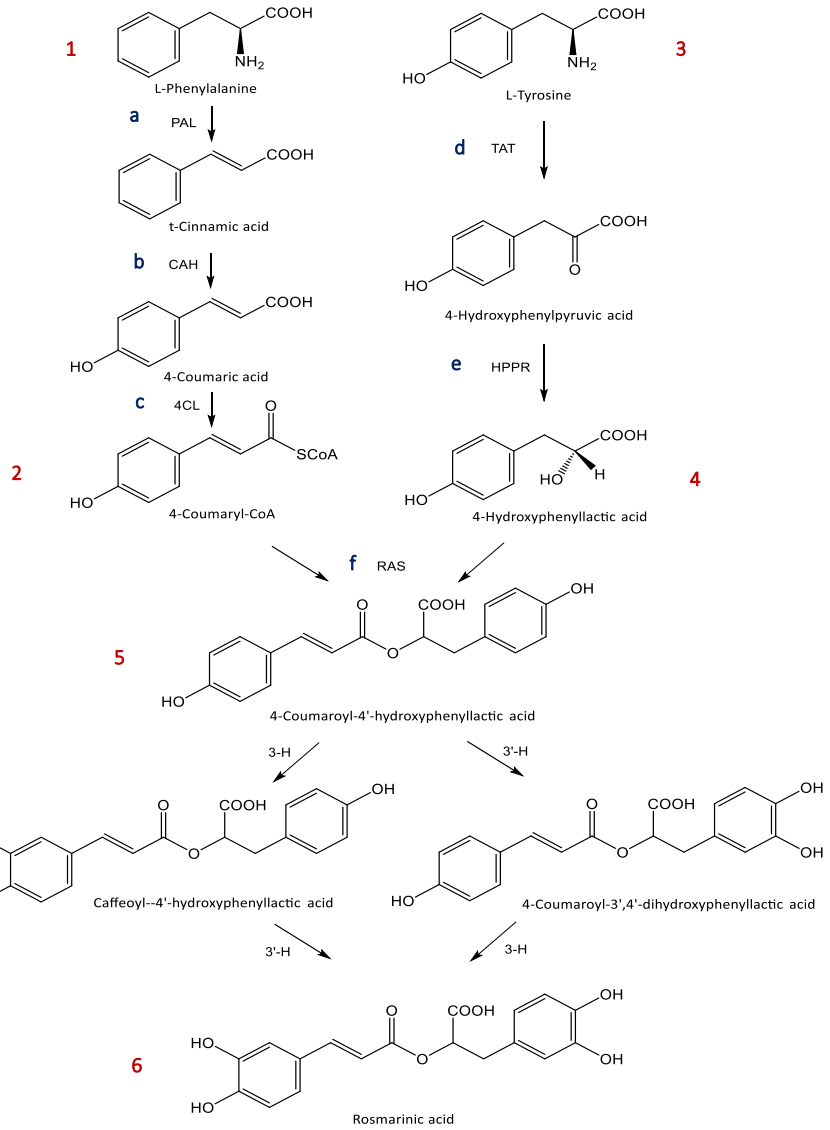


Figure A1. Biosynthetic pathway for rosmarinic acid (Adapted from Petersen et al., 2009).

PAL: phenylalanine ammonia-lyase, CAH: cinnamic acid 4-hydroxylase, 4CL: hydroxycinnamate:coenzyme A ligase, TAT: tyrosine aminotransferase, HPPR: hydroxyphenylpyruvate reductase, RAS: hydroxycinnamoyl-CoA:hydroxyphenyllactate hydroxycinnamoyl transferase, 3-H, 3′-H: hydroxycinnamoyl-hydroxyphenyllactate 3- and 3′-hydroxylases.

B. Analytical Methods Validation

B.1. High Performance Liquid Chromatography

B1.1. Calibration Curve

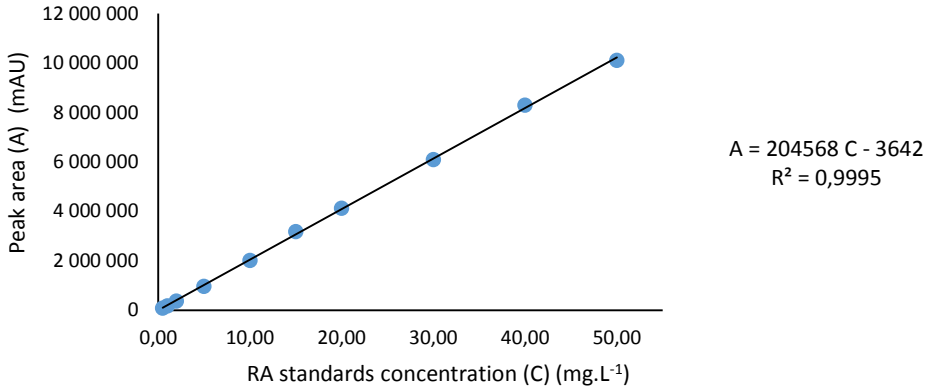


Figure A2. Calibration curve for RA quantification in aqueous solutions by HPLC.

B.1.2. Precision

Table A1. Repeatability (CV%) of the HPLC method for RA quantification at three concentration levels.

RA standard concentration (mg.L^{-1})	Repetition (n)	A (mAU)	Average A (mAU)	CV (%)
2.0	1	453557	449762	1.9
	2	461270		
	3	449008		
	4	442536		
	5	454677		
	6	437522		
15	1	3291114	3224456	2.2
	2	3284589		
	3	3293079		
	4	3161315		
	5	3161121		
	6	3155520		
40	1	8263001	8287361	2.2
	2	8294719		
	3	7957016		
	4	8416748		
	5	8311104		
	6	8481576		

Table A2. Intermediate precision (CV%) of the HPLC method for RA quantification at three concentration levels.

Day	Repetition (n)	2 mg.L^{-1}			15 mg.L^{-1}			40 mg.L^{-1}		
		A (mAU)	CV (%)	Average CV (%)	A (mAU)	CV (%)	Average CV (%)	A (mAU)	CV (%)	Average CV (%)
1	1	375126	0.8	0.6	3162110	1.4	0.7	8320615	0.6	1.0
	2	372278			3217069			8279939		
	3	369016			3126498			8377044		
2	1	388380	0.5		3191497	0.5		8213514	0.2	
	2	387624			3180203			8188119		
	3	391480			3213195			8205964		
3	1	453557	0.5		3291114	0.1		8263001	2.3	
	2	451270			3284589			7957016		
	3	449008			3293079			8294719		

B.2. UV-Vis Spectrometry

B.2.1. Calibration Curves

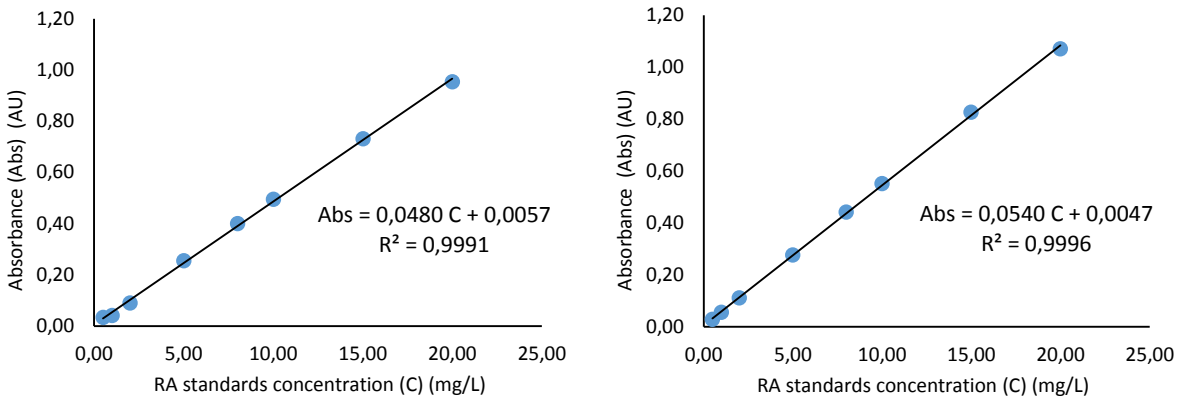


Figure A3. Calibration curve RA quantification in water (a) and coconut oil (b) by UV-Vis spectrometry.

B.2.2. Precision

Table A3. Repeatability (CV%) of the UV-Vis Spectrometry method for RA quantification at three concentration levels.

Medium	RA standard concentration ($mg.L^{-1}$)	Repetition (n)	Abs (AU)	Average Abs (AU)	CV (%)
Water	1	1	0.0400	0.0423	4.9
		2	0.0430		
		3	0.0416		
		4	0.0410		
		5	0.0421		
		6	0.0460		
	8	1	0.3990	0.3885	3.4
		2	0.4001		
		3	0.3900		
		4	0.3634		
		5	0.3897		
		6	0.3890		
	15	1	0.7305	0.7358	0.5
		2	0.7326		
		3	0.7380		
		4	0.7368		
		5	0.7395		
		6	0.7375		
Coconut oil	1	1	0.0280	0.0282	5.0
		2	0.0271		
		3	0.0286		
		4	0.0263		
		5	0.0285		
		6	0.0304		
	8	1	0.4382	0.4381	4.0
		2	0.4436		
		3	0.4209		
		4	0.4156		
		5	0.4627		
		6	0.4477		
	15	1	0.8233	0.8300	1.1
		2	0.8289		
		3	0.8196		
		4	0.8307		
		5	0.8462		
		6	0.8314		

Table A4. Intermediate precision (CV%) of the UV-Vis spectrometry method for RA quantification at 3 concentration levels.

Medium	Day	Repetition (n)	1 mg.L^{-1}			8 mg.L^{-1}			15 mg.L^{-1}		
			A (mAU)	CV (%)	Average CV (%)	A (mAU)	CV (%)	Average CV (%)	A (mAU)	CV (%)	Average CV (%)
Water	1	1	0.0400	3.6	1.5	0.3990	1.40	1.8	0.7305	0.53	0.4
		2	0.0430			0.4001			0.7326		
		3	0.0416			0.3900			0.7380		
	2	1	0.0829	0.2		0.4961	0.12		0.4961	0.12	
		2	0.0827			0.4973			0.4973		
		3	0.0825			0.4967			0.4967		
	3	1	0.0766	0.7		0.3631	4.03		0.8074	0.64	
		2	0.0777			0.3894			0.8174		
		3	0.0774			0.3900			0.8099		
Coconut oil	1	1	0.0280	2.6	2.7	0.4382	2.73	1.7	0.8233	0.57	0.5
		2	0.0271			0.4436			0.8289		
		3	0.0286			0.4209			0.8196		
	2	1	0.0183	3.0		0.4201	1.19		0.8022	0.62	
		2	0.0187			0.4295			0.7989		
		3	0.0194			0.4217			0.7925		
	3	1	0.0301	2.4		0.4476	1.29		0.8334	0.24	
		2	0.0298			0.4508			0.8298		
		3	0.0312			0.4589			0.8301		

C. Antioxidant Activity

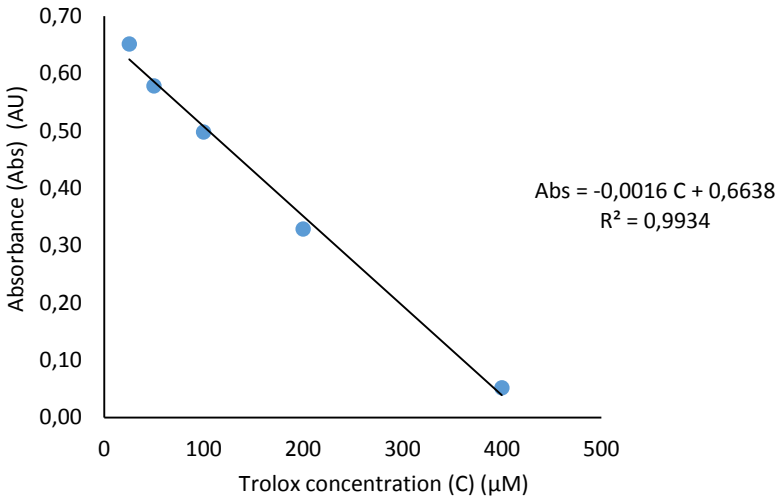


Figure A4. Linear regression for antioxidant activity estimated by ABTS method.

D. Poster Presentations

Poster presented to 8th Meeting of Young Researchers from U.PORTO, in May 2015

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Laboratory for Process Engineering, Environment, Biotechnology and Energy

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Encapsulation of a Natural Bioactive Compound for Industrial Applications

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Introduction

Natural matrices represent a rich source of bioactive compounds, having a recognized potential for the development of pharmaceutical, cosmetic and food products due to their strong activities and non-toxicity [1, 2]. Topically applied antioxidants constitute important cosmetic active ingredients capable of both protecting skin cells against the damaging effects of reactive species, as well as protecting the cosmetic formulation against oxidative degradation [3, 4].

In particular, rosemary (*Rosmarinus officinalis*) (Figure 1), an herb native to the Mediterranean region (including Portugal), possesses very useful antioxidant properties, which appears to be related to their content of phenolic compounds, amongst which rosmarinic acid (RA) was found to be one of the most important [5]. However RA (Figure 2) instability, poor solubility in water (1 mg/L) and low partition coefficient (log Kow = 1.82) constrain its transport across biological barriers and the inclusion in a cosmetic formulation [6].

These issues can be solved by applying the microencapsulation technology (Figure 3), which provides the required technique for conversion of the antioxidant to an effective functional ingredient and allows its direct and controlled delivery. One of the most used encapsulation method is the spray-drying process, due to its low cost, availability of equipment and efficiency [8]. Biopolymers and biodegradable polymers, such as chitosan, are the encapsulating materials with greater interest for pharmaceutical, cosmetic and food applications [9].

The purpose of this study was to prepare and characterize chitosan and modified chitosan microparticles encapsulating RA by a spray drying technique, as means to overcome its limitation in cosmetic formulations, as well as to study the controlled release of RA under simulated conditions.

Figure 1. Rosemary (*Rosmarinus officinalis*).

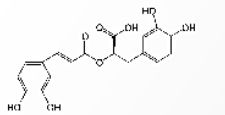


Figure 2. Rosmarinic acid chemical structure.

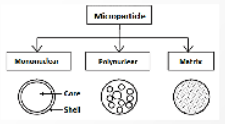


Figure 3. Morphology and scheme of microparticles.

Experimental

Preparation of rosmarinic acid-loaded microparticles: Spray Drying Process

20 mL of rosmarinic acid solution (1 g/L)
100 mL of encapsulating agent solution (10 g/L):
- Chitosan
- Modified chitosan (water soluble chitosan)

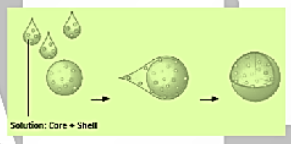
→ Mix of solutions: 540 rpm, 30min → Spray Dryer

Figure 4. Spray Drying equipment.

Spray Drying Conditions

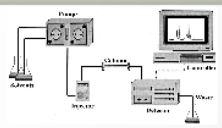
Spray-dryer BÜCHI B-290
- Nozzle: 0.5 mm
- Flow rate: 4 mL.min^{-1} (15%)
- Air pressure: 6.0 bar
- Inlet temperature: 115 °C
- Outlet temperature: 60 °C

Spray Drying Process



Analytical Methods

HPLC/DAD



- Stationary phase: Purospher STAR RP-18 endcapped (5 µm) LiChroCART 250 4
- Mobile phase: 75% citric acid in water (10 mM); 25% acetonitrile
- Flow rate: 0.8 mL.min^{-1}
- Injection volume: 100 µL
- Detection: 332 nm
- Temperature: ≈ 20 °C

UV-Vis Spectroscopy

Figure 5. UV-Vis Spectroscopy equipment.

Jasco V-530 UV/VIS Spectrophotometer
- Detection: 327 nm

Results

Rosmarinic acid-loaded microparticles

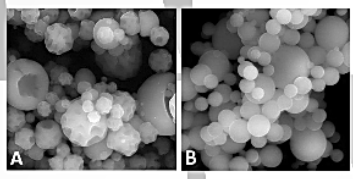


Figure 6. SEM image: Detail of the microparticles prepared with chitosan (A) and modified chitosan (B).

Product yield:
- Modified chitosan: 39,76%
- Chitosan: 42,57%

Particles mean diameter: 3 µm

Morphology:
- Modified chitosan: smooth surface
- Chitosan: rough surface

Analytical Methods

HPLC/DAD

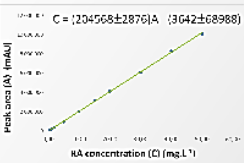


Figure 7. Calibration curve for rosmarinic acid quantification in aqueous solutions

- Linear range: 0.5-50 mg.L^{-1}
- R^2 = 0,9995
- LOD = 0.0004 mg.L^{-1}
- LOQ = 0.0012 mg.L^{-1}
- Repeatability
  - 2 mg.L^{-1}: CV = 0.09%
  - 15 mg.L^{-1}: CV = 2.22%
  - 40 mg.L^{-1}: CV = 2.19%
- Intermediate precision
  - 2 mg.L^{-1}: CV = 0.62%
  - 15 mg.L^{-1}: CV = 0.70%
  - 40 mg.L^{-1}: CV = 1.01%

Microparticles caused HPLC column blocking and damage even with successive washing steps and the controlled release results were not reliable

→ **UV-Vis Spectroscopy**

References

[1] Barbosa M.B., Ferreira I., Soares M., Carvalho A.V., Santos-Buelga C., Fernandes I.P., Barreiro M.F., Ferreira I.C.F.R., Industrial Crops and Products, 58 (2014) 230.
[2] Celiktas-Yesilyurt A., Stojanovic Z., Manojlović V., Nerdovic V., Jadranin M., Vidović S., Gavarić N., Bozin B., Food Research International, 44 (2011) 1204.
[3] Aziz M.G., Mukhtar H., Journal of Cosmetic Science, 55 (2004) 19.
[4] Carrillo J.D., Tena M.T., Recent Research Developments in Cosmetic Science, 2 (2000) 65.
[5] Hernández-Hernández E., Ponce-Alquicira E., Jaramillo-Flores M.E., Guerrero L.G., Meléndez-Herrera E., Food Chemistry, 81 (2003) 713.
[6] Soto S.B., Amorim M., Fortes P., Mascarenhas R., Ferreira D., Pintado M., Sarmento B., Pharmaceutical Biology, 51 (2013) 342.
[7] Kim H.J., Kim I.H., Kang K.S., Park J.D., Jeong H.J., International Journal of Cosmetic Science, 22 (2000) 265.
[8] Fávaro-Trindade C.S., Rocha F., Santos J., Rocha A., Trends in Food Science & Technology, 19 (2008) 384.
[9] Ammala A., International Journal of Cosmetic Science, 35 (2013) 113.

Conclusions

We concluded that it is possible to encapsulate rosmarinic acid using different types of chitosan, through a spray-drying process. Microparticles of RA with 3 µm were obtained for the biopolymers tested. From the performed tests, the results are significant and prove the success of the RA microencapsulation for a topical delivery. Controlled release studies of RA under simulated conditions by UV-Vis spectroscopy still need to be performed.

Acknowledgements

This work was funded by FEDER funds through the Operational Programme for Competitiveness Factors – COMPETE, ON.2 - O Novo Norte - North Portugal Regional Operational Programme and National Funds through FCT - Foundation for Science and Technology under the projects: PEst-C/EQB/UI0511, NORTE-07-0124-FEDER-000025 - RL2_ Environment & Health.

Abstract of Poster to be presented at 6th Workshop on Green Chemistry and Nanotechnologies in Polymer Chemistry

ENCAPSULATION OF ROSMARINIC ACID INTO CHITOSAN AND MODIFIED CHITOSAN MICROPARTICLES FOR TOPICAL DELIVERY

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Introduction

Topically applied antioxidants constitute important cosmetic active ingredients capable of both protecting skin cells against the damaging effects of reactive species, thus preventing skin aging and damage (including skin cancer), as well as protecting the cosmetic formulation against oxidative degradation. Recently, interest has increased considerably in finding naturally occurring antioxidants for cosmetic applications to replace synthetic antioxidants, which are being restricted due to their toxicity and side effects, e.g. carcinogenic effects [1, 2].

Natural matrices represent a rich source of biologically active compounds, such as antioxidants, vitamins and minerals, having a recognized potential for the development of cosmetic products [3]. In particular rosemary (Rosmarinus officinalis) extracts, herb native to the Mediterranean region in countries including Portugal, possess very useful antioxidant properties, which appear to be related to their content of phenolic compounds, amongst which rosmarinic acid was found to be one of the most important [4].

Rosmarinic acid (RA) is a naturally occurring phenolic compound commonly found in plants belonging to the Boraginaceae family and the subfamily Nepetoideae of the Lamiaceae family [5]. RA as number of interesting biological activities, including anti-inflammatory, anti-allergic, antimicrobial, antiviral, anxiolytic, neuro-protective, amongst others [6, 7, 8]. However the most important activities of rosmarinic acid are the high antioxidant activity and the anti-carcinogenic effects. RA helps to prevent skin aging and damage caused by free radicals, thereby reducing the risk of cancer [5]. Rosmarinic acid is therefore a compound of interest for cosmetic applications. However compatibility and stabilization are major concerns. Since antioxidants can be very unstable, they may become oxidized and inactive before reaching the target body site, hence the efficacy and benefit of an antioxidant is very much dependent on the delivery of the antioxidant to the organism. Transdermal delivery of rosmarinic acid through cosmetic formulations is a challenge due to various reasons, including instability, discoloration, poor solubility, low partition coefficient and poor absorption, constraining the transport across biological barriers and the inclusion in a cosmetic formulation [9].

These issues can be solved by applying the microencapsulation technology, which provides the required technique for conversion of the antioxidant to an effective functional ingredient [8]. There are several techniques of microencapsulation, but one of the most used is the spray-drying process due to its low cost, availability of equipment and efficiency [10].

Therefore, in this work we pretend to microencapsulate rosmarinic acid with chitosan and modified chitosan by a spray-drying process and also to study the controlled release of RA under simulated conditions.

Experimental

Microencapsulation of the 20 mL of RA solution (1g/L) in 100 mL of each encapsulating agent solutions (10 g/L) was performed using a spray-dryer BÜCHI B-290 advanced (Flawil, Switzerland) with a standard 0.5 mm nozzle. The solutions containing the RA were spray-dried, under the following conditions: solution flow rates, air pressure and inlet temperature were set at 4 mL/min (15%), 6.0 bar and 115 ºC, respectively. The outlet temperature is a consequence of the other experimental conditions and also of the solution properties and was around 60 ºC.

The prepared microparticles were characterized (surface and size) by scanning electron microscopy (SEM).

The release of RA from the obtained microparticles at different pH´s was accomplished by evaluating the RA content using HPLC/DAD analysis. Samples were taken at defined time intervals and analysis was performed on Merck Hitachi Elite LaChromatogragh (Tokyo, Japan) with a quaternary system of pumping (L-2130) which is equipped with LiChroCART® RP-18 end-

capped (250x4 mm, 5 µm) column attached to a guard column (4x4 mm, 5 µm) of the same type and L-2200 auto sampler with L-2455 UV/vis spectrophotometry diode array detector at 332 nm. Mobile phase composition was eluent A: 10 mM citric acid solution, acidity adjusted to pH 2.6 and eluent B: acetonitrile. Injected volume was 100 μL and the flow rate of analysis was 0.8 mL/min. The main validation parameters of the analytical method for RA were also determined.

Results and discussion

A product yield (mass ratios between the recovered powder and the quantity of raw materials used) ranging from 39.76% to 42.57% were obtained for the microparticles prepared with modified chitosan and chitosan, respectively. Microparticles with a mean diameter around 4 µm have been observed, for all the biopolymers tested (chitosan and modified chitosan). The microparticles formed with modified chitosan presented a very smooth surface, on the other hand the microparticles prepared with chitosan show a very rough surface (Fig. 1).

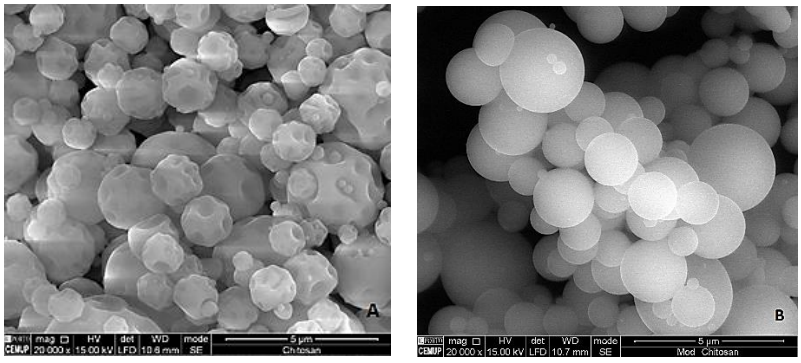

Fig.1. SEM image: Detail of the microparticles prepared with chitosan (A) and modified chitosan (B).

The quantification and the release of RA from the microparticles was evaluated by a methodology validated for this objective. A linear regression was obtained for a concentration range (0.5-50 mg/L) with a correlation coefficient (R^2) of 0.9995. The limit of detection (LOD) was 0.0004 mg/L. The method exhibited high repeatability as intra-day precisions of 0.99, 2.22 and 2.19% were achieved for low (2 mg/L), medium (15 mg/L) and high (40 mg/L) standard concentrations and the inter-day precisions were 0.59, 0.16 and 2.28% for low, medium and high standard concentrations, respectively.

The controlled release studies showed different release profiles of the RA for each pH and for each encapsulating agent.

Conclusions

We concluded that it is possible to encapsulate RA using different types of chitosan, through a spray-drying process. Microparticles of RA with 3 µm were obtained for all the biopolymers tested. From the performed tests, the results are significant and prove the success of the RA microencapsulation for a topical delivery.

References

[1] M.J. Abla, A.K. Banga, International Journal of Cosmetic Science, 35 (2013) 19.
[2] J.D. Carrillo, M. T. Tena, Flavour and Fragrance Journal, 21 (2006) 626.
[3] M.R. Barroso, L. Barros, M. Dueñas, A.M. Carvalho, C. Santos-Buelga, I.P. Fernandes, M. F. Barreiro, I.C.F.R. Ferreira, Industrial Crops and Products, 53 (2014) 330.
[4] E. Hernández-Hernández, E. Ponce-Alquicira, M. E. Jaramillo-Flores, I. G. Legarreta, Meat Science, 81 (2009) 410.
[5] D. Campos, A. R. Madureira, A. M. Gomes, B. Sarmento, M. M. Pintado, Colloids and Surfaces B: Biointerfaces, 115 (2014) 109.
[6] R. Bhatt, D. Singh, A. Prakash, N. Mishra, Drug Delivery, (2014) 1.
[7] Gudzenko, Journal of Chemical and Pharmaceutical Research, 5 (2013) 40.
[8] S. B. Silva, M. Amorim, P. Fonte, R. Madureira, D. Ferreira, M. Pintado, B. Sarmento, Pharmaceutical Biology, 53 (2015) 642.
[9] H. J. Kim, T. H. Kim, K. C. Kang, H. B. Pyo, H. H. Jeong. , International Journal of Cosmetic Science, 32 (2010) 185.
[10] B. N. Estevinho, F. Rocha, L. Santos, A. Alves, Trends in Food Science & Technology Journal. 31 (2013) 138.